A Novel Gastrin Inhibitor In Sheep Abomasal Contents

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ABSTRACT

Gastrin secretion was studied *in vitro* and *in vivo* in response to pharmacological agents and chemicals, as well as abomasal parasites and microbial products. The causes and effects of hypergastrinaemia, along with bacterial numbers and the presence of a gastrin secretion inhibitor in the abomasal contents of sheep infected with *Ostertagia circumcincta* were studied.

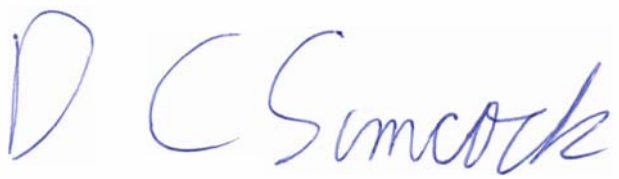
The pharmacology of the gastrin secretion from the unparasitised antrum was shown to be similar to that in monogastric animals. *In vitro* gastrin secretion by ovine antral segments was stimulated by Gastrin Releasing Peptide, carbachol and nicotine, but not adrenaline. Basal gastrin release was unaffected by somatostatin or Vasoactive Intestinal Polypeptide, but these reduced the gastrin response to stimulants. Gastrin secretion was also stimulated by amino acids, ammonia and acetate.

Hypergastrinaemia during *O. circumcincta* infection did not correlate well with decreased food intake or appear to affect parietal cell recovery. Serum gastrin concentrations correlated well with abomasal pH following adult *O. circumcincta* transplant, but poorly after larval infections. This suggests that other factors, such as inflammation and tissue damage, also affect gastrin secretion during abomasal parasitism. Anaerobic bacterial numbers in abomasal contents increased to near rumen levels when abomasal pH was 3.5 and above, but this did not affect serum gastrin concentrations. An inhibitor of *in vitro* gastrin secretion also started to appear in abomasal contents of pH3.5 and over, but did not have significant effects on *in vitro* gastrin secretion unless contents were pH4.5 and over. However, gastrin inhibitory activity in abomasal contents and serum gastrin levels were positively correlated, suggesting abomasal gastrin inhibitory activity has little effect on gastrin secretion *in vivo.*

Three competing factors were present in rumen fluid and rumen incubates: an inhibitor and a stimulant of secretion and an elimination factor. The stimulant was resistant to acid degradation, had a molecular weight below 3000 M_r and was hydrophilic. Both the elimination factor and the inhibitor were sensitive to acidity and hydrophobic and are likely to be proteinaceous.

STATEMENT

This is to certify that the work on which this thesis is based was carried out by the undersigned, and has not been accepted in whole or in part for any other degree or diploma. Assistance is specifically recorded in the Acknowledgements section bound with this thesis.



David Crispin SIMCOCK.
(2000)

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LIST OF ABBREVIATIONS

α_2	Alpha adrenergic receptor subtype 2
Ach	Acetylcholine
ANOVA	Analysis of Variance
API	Adult Parasite Infected
$\beta_{2/3}$	Beta adrenergic receptor subtype 2 or 3
BRS-3	Bombesin receptor subtype 3
BSA	Bovine Serum Albumin
cAMP	cyclic Adenosine Monophosphate
°C	Degrees Celsius
CCK	Cholecystokinin
CCK_A	Cholecystokinin receptor class A
CCK_B	Cholecystokinin/gastrin receptor class B (gastrin receptor)
cells.mL^{-1}	viable cells per millilitre
CGRP	Calcitonin Gene Related Peptide
circ	circulation
cm	centimetre
cpm	counts per minute
CTR	control
D cell	somatostatin cell
DMPP	1,1-dimethyl-4-phenylpiperrazinium
EC	Enterochromaffin
ECL	Enterochromaffin like
EGF	Epidermal Growth Factor
e.p.g	eggs per gram
E/S	Excretory/Secretory
FEC	Faecal Egg Counts
g	grams
g	g force
g.L^{-1}	grams per litre
G17	Gastrin-17
G34	Gastrin-34
G17Gly	Glycine extended G17
G34Gly	Glycine extended G34
G-Gly	Glycine extended gastrins
G cell	Gastrin cell
GABA	Gamma Amino Butyric Acid
GIP	Gastric Intestinal Polypeptide
GLP	Glucagon Like Peptide
GRP	Gastrin Releasing Peptide
$GRPR_1$	GRP receptor subtype 1
$H_{2/3}$	Histamine receptor subtype 2 or 3
HBSS	Hank's Balanced Salt Solution
H. contortus	*Haemonchus contortus*
H. pylori	*Helicobacter pylori*
hGRP	human GRP
I cells	cholecystokinin containing cells
IL	Interleukin
INF	Interferon Gamma

iU	international unit
kDa	kiloDalton
kg	Kilogram
L	Litre
L3	Third stage larvae
LPI-1/2	Larval parasite infected, experiment 1 or 2
LTI	Larval Trickle Infected
M	Moles per litre
$M_{1/2/3}$	cholinergic receptor subtype 1, 2 or 3
mg	milligram
$mg.mL^{-1}$	milligrams per millilitre
$mg.kg^{-1}$	milligrams per kilogram
mL	millilitre
$mL.L^{-1}$	millilitres per litre
$mL.min^{-1}$	millilitres per minute
mM	millimolar
mOsm	milliosmoles
$mOsm.L^{-1}$	milliosmoles per litre
$\mu g.kg^{-1}$	micrograms per kilogram
µm	micrometres
µM	micromolar
µL	microlitre
µg	microgram
M_r	Molar ratio
mRNA	messenger RNA
NA	noradrenaline
NK	Neurokinin
NY	Neuropeptide Y
O. ostertagi/ circumcincta	*Ostertagia* spp.
p	probability statistic
PAM	Peptidylglycyl Amidating Mono-oxygenase enzyme
PBS	phosphate buffered saline
PC	Prohormone Convertase
pGRP	porcine GRP
PGE_2	Prostaglandin E_2
PHI	Peptide Histidine Isoleucine
pM	picomolar
PYY	Peptide YY
RIA	Radioimmunoassay
S. bovis	Streptococcus bovis
S cells	secretin containing cells
SD	standard deviation
SEM	standard error of the mean
SP	Substance P
SS	somatostatin
SST	somatostatin receptor
T. colubriformis	*Trichostrongylus colubriformis*
TGFα	Transforming Growth Factor Alpha

TNFα	Tumour Necrosis Factor Alpha
VIP	Vasoactive Intestinal Polypeptide
UNIANOVA	Univariate Analysis of Variance
v/v	volume to volume
w/v	weight to volume

PREFACE

Gastrin is a hormone secreted by the antrum of the stomach in monogastrics or the abomasum in ruminants. The classical action of gastrin is the control of acid secretion, for which it is the integration point for many stimulants and inhibitors. Gastrin has additional roles, notably the maintenance of gastric gland architecture and regulation of gastrointestinal motility. Hypergastrinaemia has been extensively studied in gastric diseases, particularly in humans with *Helicobacter pylori* infection, duodenal ulcers and pernicious anaemia. In duodenal ulcer patients, hypergastrinaemia is associated with the hypersecretion of acid. While most studies of gastrin secretion have been conducted in monogastric animals, the ruminant abomasum has similar architecture and functional cells to the stomach in other mammals (Murray *et al.*, 1970; Gurnsey *et al.*, 1985; Wathuta *et al.*, 1986) and its secretions also appear to be controlled by similar mechanisms (Lawton, 1995).

There is still debate regarding the causes and roles of hypergastrinaemia during abomasal nematode infection in ruminants. Fox *et al.* (1989a, b; 1993) have shown that in calves infected with *Ostertagia ostertagi*, hypergastrinaemia is very closely related to abomasal hypoacidity and a reduction in food intake. In fact, hypergastrinaemia is so closely correlated with the pathology of abomasal infection that it has been proposed as a diagnostic marker for parasitism. However, in sheep infected with *O. circumcincta*, hypergastrinaemia is not as closely related to abomasal hypoacidity. Notably, Lawton *et al.* (1996) observed that although hypergastrinaemia and abomasal hypoacidity develop in tandem, hypergastrinaemia persists when abomasal pH returns to normal levels. Thus, questions remain concerning the importance of other factors in stimulating gastrin secretion in the parasitised abomasum.

A particularly unusual observation in some parasitised sheep was a reversal of the hypergastrinaemia when abomasal pH exceeded pH5.5. This was suggested to be due to abomasal microbes inhibiting gastrin secretion. Microbial involvement in gastrin secretion during parasitism was supported by studies *in vitro*, in which a potent inhibitor of gastrin secretion was produced

when abomasal microbes were incubated aerobically (Haag, 1995; Lawton, 1995). This effect appears to be novel, as similar effects have not been reported in the numerous studies of *H. pylori* infections. The principal inhibitor of gastrin secretion in all species studied is somatostatin. Lawton (1995) suggested that the microbial inhibitor of gastrin secretion may be a somatostatin-like substance, however, the inhibitor reduced basal gastrin secretion in the *in vitro* antral preparation, unlike endogenous somatostatin released by pharmacological agents. Thus, it seems likely that the microbial factor which inhibited gastrin release appeared to act via a novel mechanism.

The primary objective was to determine the characteristics of the microbial inhibitor of gastrin secretion and whether it affected gastrin secretion during abomasal parasitism. To examine this, gastrin secretion was studied *in vitro* using tissue cultures and *in vivo* in sheep parasitised with *O. circumcincta*. *In vivo* studies were also used to examine the abomasal bacterial numbers and hypergastrinaemia during abomasal parasitism, as well as possible effects of hypergastrinaemia. *In vitro* experiments were also used to determine properties of the microbial inhibitory activity, and whether it was present in abomasal and rumen contents. *In vitro* and *in vivo* studies were combined to determine whether the microbial inhibitor of gastrin secretion affects gastrin levels during abomasal parasitism.

CHAPTER 1

LITERATURE REVIEW

The study of gastrin has a long history (reviewed by Dockray and Gregory, 1989), beginning in 1905 when Edkins first proposed that a pyloric hormone controlled gastric secretion. This was proven by Grossman *et al.* (1948) and the first gastrin peptide was isolated by Gregory and Tracy (1964). Sequencing of the different molecular forms of gastrin in many species, including sheep (Agarwal *et al.,* 1968), rapidly followed.

The development of radioimmunoassays for gastrin (McGuigan, 1968; Hansky and Cain, 1969; Yalow and Berson, 1971) allowed the specific assay of secreted gastrin. During the 1970s, most experiments on gastrin were conducted *in vivo* and focused on the role of gastrin in the control of acid secretion (reviewed by Walsh and Grossman, 1975a, b; Dockray and Gregory, 1989). Saffouri *et al.* (1979, 1980) first reported the functional link between antral gastrin and somatostatin secretion, beginning a period of experiments using *in vitro* methods which have helped clarify the control of gastrin secretion by hormones, nerves and chemicals (reviewed by Schubert, 1994, 1997, 1998). The connection between *Helicobacter pylori,* hypergastrinaemia and duodenal ulcers (Levi *et al.,* 1988) has led to studies on the effects of inflammation on gastrin secretion.

In parallel, the development of region-specific antisera allowed the biosynthesis of gastrin to be determined (reviewed by Dockray *et al.,* 1996; Dickinson *et al.,* 1997; Dockray, 1999). The secretion and bioactivity of the glycine-extended precursors of amidated gastrins have also been studied using specific antisera. Once thought to be inactive, these peptides have been shown to have significant trophic actions. Recently, molecular biological techniques and transgenic mice have been used to explore cell signalling pathways, the activity of gastrin receptors and the physiological roles of gastrin (reviewed by Dockray, 1999; Hinkle and Samuelson, 1999).

The control of gastrin secretion is discussed in this chapter which is divided into two parts. In the first, the mechanisms controlling gastrin secretion

are discussed. In the second, gastrin secretion during abomasal parasitism is reviewed. The concentration of gastrin in the circulation is dependent upon both the rates of gastrin secretion and degradation after secretion. Gastrin release is itself controlled by the number of G cells in tissue, the production of gastrin in these cells and the secretion of gastrin from these cells. This review focuses particularly on the control of antral G cell numbers, their production and release of gastrin, the post-secretory metabolism of gastrin and the mechanisms regulating antral gastrin secretion.

1.1 Gastrin: bioactivity, location, synthesis and secretion

1.1.1 Bioactivity of gastrins

The classical effects of amidated gastrins are the stimulation of acid secretion and fundic mucosal growth (Schubert, 1994). In monogastric animals, gastrin also stimulates gall bladder contraction (Gregory and Tracy, 1964; Valenzuela *et al.,* 1976), gastrointestinal motility (Gregory and Tracy, 1964; Strunz *et al.,* 1979) and pancreatic secretion (Valenzuela *et al.,* 1976). In ruminants, gastrin slows reticuloruminal contractions (Carr *et al.,* 1970; Ruckebusch *et al.,* 1971; Nicholson *et al.,* 1982; McLeay and Wong, 1989), omasal emptying (Onapito *et al.,* 1978) and abomasal emptying (Ruckebusch, 1971; Bell *et al.,* 1975, 1977), but stimulates abomasal contractions (McLeay and Wong, 1989). Although some of these actions of gastrin were demonstrated with supra-physiological doses, they may still occur *in vivo* when combined with other effectors (Grossman, 1970). Gastrin has been reported to have cytoprotective effects on the gastric mucosa (Bacha *et al.,* 1997; Mercer *et al.,* 1998).

In some cases, gastrin and cholecystokinin have overlapping bioactivity. For example, both gastrin and cholecystokinin stimulate pepsinogen secretion, although gastrin is less potent than cholecystokinin in this action (Hersey *et al.,* 1983; Kleveland *et al.,* 1987; Cherner *et al.,* 1988; Lin *et al.,* 1992; Lanas *et al.,* 1994; Blandizzi *et al.,* 1999). Similarly, both cholecystokinin and gastrin reduce

food intake. Many studies have shown cholecystokinin to reduce appetite (reviewed by Fink *et al.,* 1998; Ritter *et al.,* 1999); gastrin also inhibits food intake via a central mechanism in neonatal chicks (Furuse *et al.,* 1999), and hypergastrinaemia is associated with decreased food intake in cattle (Fox *et al.,* 1989a; Fox, 1997). In addition, gastrin stimulates the production and secretion of leptin from adipose tissue (Attoub *et al.,* 1999). Leptin is thought to be involved in mediation of long term appetite, but can also interact with cholecystokinin to suppress short term food intake (Barrachina *et al.,* 1997).

The active site of both gastrin and cholecystokinin peptides is the same amidated C-terminal pentapeptide sequence. Otherwise, gastrin and cholecystokinin have different amino acid sequences, a critical difference being the position of the C-terminal tyrosyl residue and its level of sulphation (Rehfeld, 1981).

The bioactivities of the amidated gastrins, glycine-extended gastrins (G-Gly) and cholecystokinin are determined by their affinity for the different cholecystokinin/gastrin receptors. Amidated gastrins act on CCK_B/gastrin (CCK_B) receptors only, whereas cholecystokinin binds to both the CCK_A and CCK_B receptors (DelValle *et al.,* 1993; Zavros *et al.,* 1997). Blockade of CCK_A receptors converts cholecystokinin from a partial to a full gastrin-like agonist of acid secretion (Konturek and Konturek, 1993; Schmidt *et al.,* 1994). A sequence of 5 amino acids in the CCK_B receptor is essential for gastrin binding (Silvente-Poirot and Wank, 1996), but only the amidated C-terminal pentapeptide sequence of gastrin and cholecystokinin is essential for gastrin receptor activation (Walsh and Grossman, 1975a). G-Gly act via novel CCK/gastrin receptors (Baldwin, 1994; Seva *et al.,* 1994; Singh *et al.,* 1995; Hollande *et al.,* 1997) and while able to bind to the CCK_B receptor, G-Gly peptides are 100 times less potent agonists than amidated gastrins (Matsumoto *et al.,* 1987; Sandvik and Dockray, 1999).

1.1.1.1 Amidated gastrins

Amidated gastrins stimulate acid secretion by binding to CCK_B receptors. In the stomach, these are present on parietal cells and fundic enterochromaffrin-like (ECL) cells (Song *et al.,* 1996; Kinoshita *et al.,* 1998), as well as antral D cells (Song *et al.,* 1996; Helander *et al.,* 1997; Kinoshita *et al.,*

1998) and fundic D cells (DelValle *et al.,* 1993; Song *et al.,* 1996; Helander *et al.,* 1997; Zavros *et al.,* 1997, 1998). Gastrin stimulates acid secretion mainly by stimulating fundic histamine release and production (Hocker *et al.,* 1998), but also has direct effects on the parietal cell. Somatostatin, release of which is stimulated by gastrin, inhibits both gastrin and acid secretion and thus forms a 'negative feedback loop' with gastrin (Schubert, 1997). Amidated gastrin and the CCK_B receptor are also essential for the maturation of the parietal cell. Deletion of either the gastrin or CCK_B receptor gene in transgenic mice eliminates basal acid secretion and renders the parietal cell unresponsive to its classical stimulants (Nagata *et al.,* 1996; Koh *et al.,* 1997; Langhans *et al.,* 1997; Friis-Hansen *et al.,* 1998).

Amidated gastrin is a trophic factor for the fundus, increasing mucosal thickness and stimulating parietal cell differentiation and ECL cell proliferation (reviewed by Dockray, 1999). As the ECL cell is self-replicating, the trophic effects of gastrin are probably direct (Tielemans *et al.,* 1989; Fukui *et al.,* 1998; Kinoshita *et al.,* 1998). In contrast, parietal cells must be affected by gastrin indirectly, as mature parietal cells cannot divide (Karam, 1993) while their precursors, which can divide, do not express the CCK_B receptor (Fukui *et al.,* 1998). The Reg1-α protein has been suggested to mediate the trophic effects of gastrin on the parietal cell, because it is produced by both ECL cells and chief cells in humans (Asahara *et al.,* 1996; Higham *et al.,* 1999) and is upregulated by hypergastrinaemia (Fukui *et al.,* 1998; Higham *et al.,* 1999). The trophic effects of gastrin on epithelial cells may also in part be mediated by increased expression of amphiregulin and other epidermal growth factor (EGF) peptides, such as heparin binding EGF-like growth factor (Tsutsui *et al.,* 1997; Miyazaki *et al.,* 1999). Amidated gastrin also appears to have trophic effects on the colon (Wang *et al.,* 1996).

Long term hypergastrinaemia has almost opposite results to short term hypergastrinaemia, causing increased epithelial growth, gradual parietal cell loss and gastric atrophy (Wang *et al.,* 1999). Increased levels of EGF-related peptides may be responsible for the effects of long-term hypergastrinaemia (Dockray, 1999).

1.1.1.2 Glycine-extended gastrins

Glycine-extended gastrins typically act via mechanisms separate from CCK_B receptor binding. G-Gly upregulates expression of the H^+/K^+ ATPase α-subunit in canine parietal cells via a novel receptor (Kaise *et al.,* 1995). In rats, G-Gly potentiated stimulatory effects of amidated gastrins on acid secretion (Dickinson *et al.,* 1990a; Higashide *et al.,* 1996) but no such effect is noted in humans (Hilsted *et al.,* 1988b; Hansen *et al.,* 1996c). Thus, the importance of G-Gly in controlling acid secretion varies between species.

Glycine-extended gastrins have been shown to have trophic effects. Some of the previously reported trophic effects of amidated gastrin are now believed to be due to G-Gly (Dickinson *et al.,* 1997). In the colon, G-Gly stimulates mucosal growth (Dickinson, 1995; Wang *et al.,* 1996; Koh *et al.,* 1999), and may be partly responsible for the increased risk of colorectal carcinoma associated with hypergastrinaemia (Thorburn *et al.,* 1998). Carcinoma cell lines have been identified in which G-Gly stimulates growth (Koh *et al.,* 1996), DNA synthesis (Seva *et al.,* 1994) and, in some cell lines, may have an autocrine function (Watson *et al.,* 1991; Iwata *et al.,* 1996). In pancreatic and kidney cell lines which respond to G-Gly stimulation and also contain CCK_B receptors, G-Gly acts independently of, and is complementary to, CCK_B mediated effects (Todisco *et al.,* 1995; Stepan *et al.,* 1999).

1.1.2 Gastrin secreting tissues

In healthy adult mammals, the most abundant source of gastrin is the antrum of the stomach (Creutzfeldt *et al.,* 1971; Nilsson *et al.,* 1973; Malmstrom *et al.,* 1976; Vaillant *et al.,* 1979; Jonsson and Dockray, 1984; Lundell *et al.,* 1987; DelValle *et al.,* 1989), or the abomasum in ruminants (Reynolds *et al.,* 1984, 1991). Gastrin is not present in the gastric fundus (Voillemot *et al.,* 1978; Reynolds *et al.,* 1984). The duodenum also contains gastrin in monogastrics (Nilsson *et al.,* 1973; Lundell *et al.,* 1987; Simpson *et al.,* 1994) and in sheep (Reynolds *et al.,* 1984; Simpson *et al.,* 1993). Gastrin immunoreactivity has also been detected in the vagal nerve (Uvnas-Wallenstein *et al.,* 1977) and pituitary gland (Rehfeld, 1978).

The pancreas is a transient source of amidated gastrin, with production observed in foetal and neonatal rats (Brand and Fuller, 1988), dogs, cats, humans, pigs (Bardram *et al.,* 1990) and sheep (Read and Shulkes, 1993). In rats, the pancreas is a significant source of gastrin before antral gastrin expression (Larsson *et al.*, 1976). In humans, gastrin expression appears in the duodenum before the antrum, and is the major source of gastrin until after birth, when antral levels increase (Track *et al.*, 1979). The colon also produces small amounts of gastrin in foetal sheep (Ciccotosto and Shulkes, 1996).

Gastrin-producing tumours develop rarely in the stomach but more commonly in the pancreas (Kariya *et al.*, 1986) or colon (Ciccotosto *et al.*, 1995).

1.1.2.1 The antral G cell

Antral G cells are generally observed to be most numerous near the pylorus, with decreasing density towards the fundus (Cowley *et al.*, 1975; Stave and Brandtzaeg, 1976, 1978; Royston *et al.*, 1978; Voillemot *et al.*, 1978; Takahashi *et al.*, 1979). In sheep, however, G cell density was described as uniform in the antrum (Bunnett, 1984). The circumferential distribution of G cells in the antrum varies between studies. A greater density of G cells is reported along the lesser curvature in cats (Cowley *et al.*, 1975) and along the greater curvature in dogs (Takehashi *et al.*, 1979) and humans (Stave and Brandtzaeg, 1978), while uniform distribution in humans has also been reported (Stave and Brandtzaeg, 1976; Royston *et al.*, 1978). Depending upon the species, G cells may be located predominantly in the upper, middle or lower part of the antral gland (Polak, 1986). In sheep, G cells are reported to be present either throughout the thickness of the gland (Bunnett, 1984) or more numerous towards the base (Scott *et al.*, 1998a). Studies in sheep (Bunnett, 1984) and humans (Creutzfeldt *et al.*, 1971) revealed that some G cells have a tuft of microvilli on their apical border projecting into the lumen, which may be sensitive to luminal factors.

G cells originate from progenitor cells in the pyloric gland proliferative zone and are also believed to be self-replicating (Lehy and Willems, 1976; Bertrand and Willems, 1980), although self-replication was not supported by a study in hamsters (Fujimoto *et al.,* 1980). G cells take five to six days to mature

(Bertrand and Willems, 1980; Fujimoto *et al.,* 1980) and have a half-life of 10-15 days in hamsters (Fujimoto *et al.,* 1980) and 40 days in mice (Lehy and Willems, 1976).

1.1.3 G cell numbers

The antral G cell population increases in gastrin hypersecretory states, such as after re-feeding fasted rats (Bertrand and Willems, 1980), vagotomy (Delince *et al.,* 1978), or fundectomy (Alumets *et al.,* 1980; Magallanes *et al.,* 1982; Fabri *et al.,* 1989). Conversely, G cell numbers are decreased in parallel with the mucosal atrophy caused by food deprivation (Bertrand and Willems, 1980). Some reports of G cell hyperplasia during gastrin hypersecretion may be due to increased detection of existing G cells, because of their increased gastrin content, rather than hyperplasia. Truncal vagotomy induced hyperplasia is reported to be via activation of existing G cells and the rapid maturation of progenitor cells (Shimoda *et al.,* 1990).

Antral G cell numbers are controlled by somatostatin-secreting D cells, which are in turn controlled by gastric acidity. It has been shown that hypoacidity induced by truncal and fundic vagotomy coincided with G cell hyperplasia (Arnold *et al.,* 1982; Magallanes *et al.,* 1982; Blair *et al.,* 1986). Fundic vagotomy (Arnold *et al.,* 1982) and omeprazole treatment (Allen *et al.,* 1986; Larsson *et al.,* 1988) increase the G:D cell ratio. The reverse effect was observed with hyperacidity (Arnold *et al.,* 1982). G cell hyperplasia is also being reversed by D cell hyperplasia during corticosterone treatment (Xynos *et al.,* 1987). Thus, D cell numbers seem to have negative effects on G cell numbers. Arnold *et al.* (1984) proposed that hypoacidity alone does not increase G cell numbers, and reported that acid inhibition decreased G cell density. However, these findings are at variance with the majority of studies, which show that hypoacidity induces G cell hyperplasia.

Gastrin Releasing Peptide (GRP) may be trophic for G cells. In studies in rats, oral administration of bombesin caused G cell hyperplasia (Lehy *et al.,* 1983). Furthermore, hypergastrinaemia and G cell hyperplasia coincided with increased GRP release after truncal vagotomy (Shimoda *et al.,* 1995). EGF peptides have also been implicated in the proliferation of G cells (Vinter-Jensen

et al., 1995). The EGF receptor is expressed by G cells (Ford *et al.,* 1997) and responds to Transforming Growth Factor Alpha (TGFα) also, which is a potent mitogen for gastric progenitor cells (Sharp *et al.,* 1995) and is expressed in the gastric antrum (Beauchamp *et al.,* 1989).

1.1.4 Gastrin biosynthesis

Gastrin biosynthesis determines the quantity and the molecular forms of gastrin that can be secreted by the G cell in response to stimulation. Gastrin biosynthesis can be regulated at the level of transcription and peptide post-translational processing. Transcription ultimately controls the amount of gastrin available for secretion and regulates the long-term activity state of the secreting cell. Post-translational processing determines which forms of gastrin are secreted by the G cell.

There is developmental regulation of both post-translational processing and transcription of gastrin. Both amidation and enzymatic cleavages are upregulated in the antrum during foetal growth, after birth and after weaning (Marino *et al.,* 1988; Wang *et al.,* 1995).

1.1.4.1 Transcription of the gastrin gene

Gastrin gene transcription varies according to the secretory state of the cell and the ontogeny of the tissue. Developmental expression causes different tissues to secrete gastrin in foetal, neonatal, suckling and mature animals (Larsson *et al.,* 1976; Brand and Fuller, 1988; Bardram *et al.,* 1990; Read and Shulkes, 1993). In the mature animal, transcription is regulated largely by factors controlling secretion such as gastric pH and interstitial somatostatin concentration (Brand and Stone, 1988; Karnik *et al.,* 1989; Dockray *et al.,* 1991; Purewal *et al.,* 1997) and also may be stimulated by inflammation (Lichtenberger *et al.,* 1995; Dial *et al.,* 1996).

The 5' and 3' sequences controlling gastrin gene transcription are well conserved between species (Fuller *et al.,* 1987). The 5' promoter sequence contains sites controlling the developmental expression of gastrin and responses to external stimuli. The specific sites of control for gastrin gene transcription vary between tissues (Wang and Brand, 1990). The CACC site,

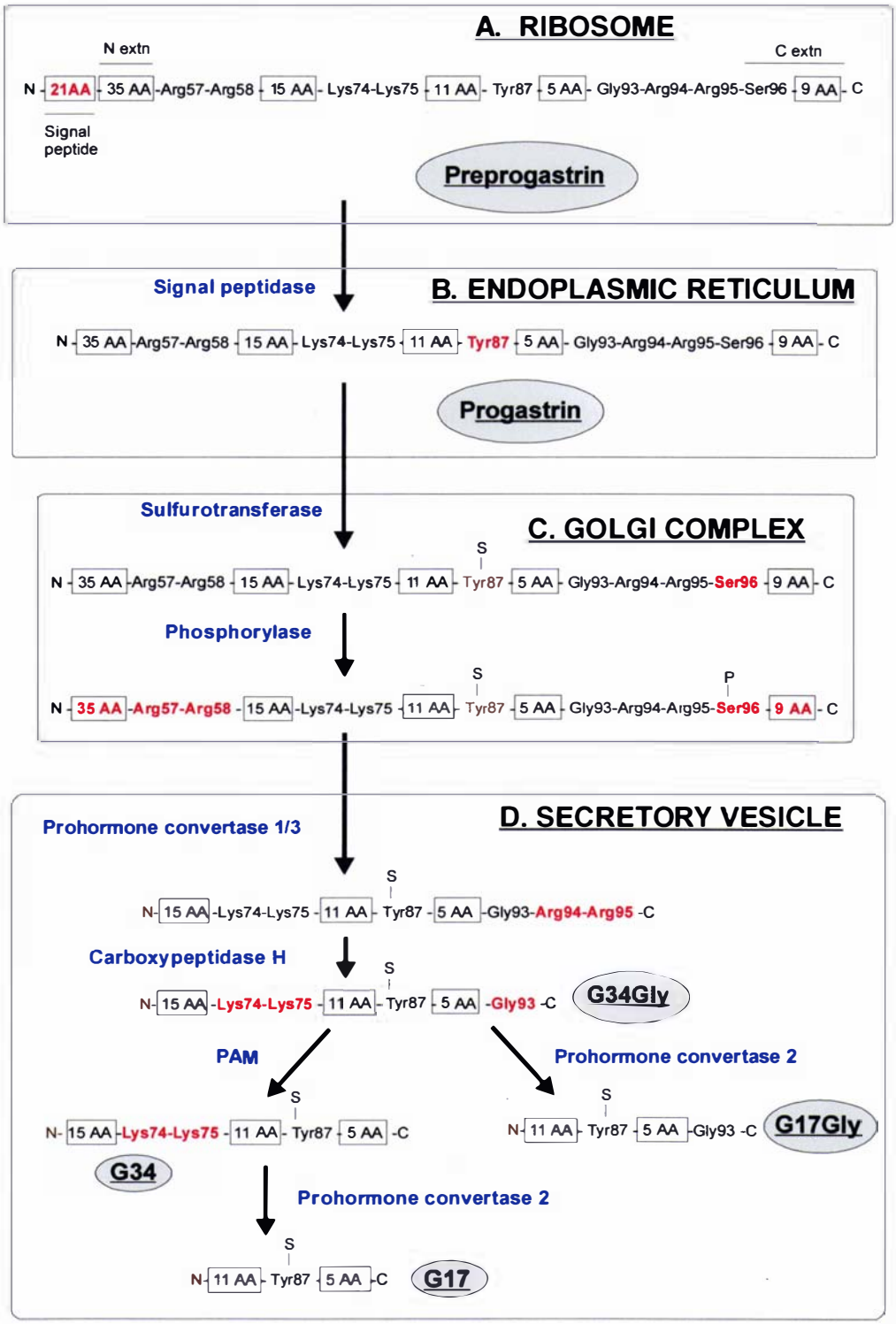

Figure 1.1. Gastrin biosynthesis in the antral G cell. Shown is the production of gastrin from the earliest peptide precursor, preprogastrin, to final amidated and glycine extended end products. Processing enzymes are labelled in blue and their sites of action in are the red parts of the gastrin sequence, with peptide names given in grey shaded areas. Abbreviations are explained in the List of Abbreviations, pages XX and XXI.

for example, is believed to be involved in the developmental regulation of gastrin gene transcription in the pancreas (Tillotson *et al.,* 1994). The CACC site is activated by a 70kDa protein in foetal tissue (Tillotson *et al.,* 1994) and inhibited by a zinc finger protein (Tillotson, 1999).

Other sites on the promoter regulate transcription in response to production and secretion stimuli. A region of the gastrin promoter called "gastrin EGF response element" (gERE) controls EGF-stimulated transcription (Merchant *et al.,* 1991, 1995, 1996) and is active in canine G cells (Ford *et al.,* 1997). The gERE site is weakly responsive to stimulation by Tumour Necrosis Factor Alpha (TNFα) (Ford *et al.,* 1997) and phorbol esters (Merchant *et al.,* 1991). In addition to gERE, there are cis-acting cyclic adenosine monophosphate (cAMP) inducible elements in the gastrin gene (Shiotani and Merchant, 1994, 1995).

As well as stimulating activity at gERE, EGF also activates immediate early response genes such as *cfos* (Dockray *et al.,* 1996; Ford *et al.,* 1997). The product of *cfos*, fos, binds other DNA proteins to upregulate transcription (Chiu *et al.,* 1988). The fos protein alone cannot stimulate the gastrin promoter, but fos is a factor required for EGF stimulation of gastrin transcription and its production is regulated by many factors, such as cytokines (Marks *et al.,* 1996). Multiple stimuli are probably required for maximum transcription activity.

Stimulation of gastrin gene expression and gastrin secretion are two discretely regulated processes. In general, specific stimulants of gastrin secretion are poor stimulants of gastrin gene transcription (Ford *et al.,* 1997). Hypersecretion of gastrin is, however, associated with upregulation of gastrin gene expression (Brand and Fuller, 1988; Wu *et al.,* 1990; Dockray *et al.,* 1991). Thus, there is some linkage between gastrin gene transcription and secretory levels, though the precise mechanisms are unknown.

1.1.4.2 Post-translational processing

Post-translational processing is outlined in Figure 1.1, which shows sequential cleavage, sulphation, phosphorylation and amidation steps involved in the formation of amidated gastrin. Sequence homology at the cleavage sites and the active C-terminal of the final peptide are well conserved between species (Fuller *et al.,* 1987). However, there are differences in the sequences

of the extensions (Jonsson and Dockray, 1984; Fuller *et al.,* 1987), which may alter the secondary structure of progastrin and thus alter post-translational processing (Fuller *et al.,* 1987).

1.1.4.2.1 Ribosomes and Endoplasmic reticulum

Gastrin mRNA is translated in the ribosome to preprogastrin (Figure 1.1A). Preprogastrin then enters the endoplasmic reticulum where the signal peptide is cleaved, forming progastrin (Figure 1.1B). This peptide is then packaged in vesicles and transported into the Golgi complex for peptide modification.

1.1.4.2.2 The Golgi Complex

Both sulphation and phosphorylation of the peptide occur in the Golgi Complex (Varro *et al.,* 1993, 1994, Figure 1.1C), but neither appears to be vital to biological activity of amidated gastrin (Dickinson *et al.,* 1997).

Sulphation occurs prior to phosphorylation (Varro *et al.,* 1994), is incomplete, and the rate may differ between tissues (Brand and Fuller, 1988). Sulphation of the amidated peptides is around 50-66% in most species, notable exceptions being dogs with 24% and sheep with 80% sulphation (Andersen, 1985). There is some debate concerning the role of gastrin sulphation in further peptide processing. Sulphation has been reported to have no effect on further processing of gastrin (Hilsted and Rehfeld, 1987) and is not affected by feeding in rat studies (Varro *et al.,* 1990b). In an endocrine cell line, however, sulphation increased endoproteolytic cleavage of the progastrin peptide (Bundagaard *et al.,* 1995).

Phosphorylation at Ser-96 occurs immediately prior to cleavage of progastrin at Arg94-Arg95 (Varro *et al.,* 1994) and may be linked to this event (Dockray *et al.,* 1987; Varro *et al.,* 1988). In insulinoma cells, which have similar processing pathways to G cells, phosphorylation and dibasic cleavage rates are inversely proportional (Bishop *et al.,* 1998). Opposite results were reported in rat antral G cells, where decreased phosphorylation was associated with decreased cellular turnover of gastrin, due to reduction of either synthesis or secretion (Dimaline *et al.,* 1991; Varro *et al.,* 1990a, b, c). It is therefore proposed that phosphorylation is only one of several factors controlling

progastrin cleavage (Bishop *et al.,* 1998). There are also species differences in the level of progastrin phosphorylation, probably due to the differing properties of enzymes involved (Desmond *et al.,* 1989).

1.1.4.2.3 The secretory vesicles

Progastrin is cleaved to form active gastrin in the secretory vesicles (Dickinson *et al.,* 1993; Dockray *et al.,* 1996; Figure 1.1D). Staining for G34 and G17 occurs only in the secretory vesicle of G cells (Rahier *et al.,* 1987).

Progastrin is converted to G34-Gly by cleavage at the C-terminus of the Arg57-Arg-58 and Arg94-Arg-95 dibasic sites by prohormone convertase (PC) 1/3 and removal of the Arg94-Arg95 by carboxypeptidase H (Dickinson *et al.,* 1995). The amino-terminal glutamine residue of G34-Gly then undergoes pyrole ring formation, which increases the resistance of the peptide to degradation in the blood (Dickinson *et al.,* 1997).

Studies in rats show that G34-Gly is the substrate for amidation (Varro *et al.,* 1995). G17-Gly appears to be a distinct end product, as it is not amidated but is stored and secreted with G17 (Varro *et al.,* 1995). Studies on the amidating enzymes of the pituitary and antrum revealed two peptidylglycyl amidating mono-oxygenase (PAM) enzymes, PAM-A and PAM-B, which require ascorbate (Sugano *et al.,* 1987b), catalase and copper for activity (Dickinson *et al.,* 1990a; Dickinson and Yamada, 1991).

Only after the G34 cleavages have occurred can G17 be formed (Dickinson *et al.,* 1992; Bishop *et al.,* 1998). The G17 molecule is formed by cleavage of G34 by the PC2 enzyme (Dickinson *et al.,* 1995), not PC1/3 (Marino *et al.,* 1991; Dickinson *et al.,* 1995), and is the slowest of the progastrin cleavages (Varro *et al.,*, 1995). Whether cells secrete G34 or G17 therefore depends upon whether they express the PC2 enzyme (Dickinson *et al.,* 1995). These prohormone convertase enzymes are likely to be responsible for gastrin production in the antral G cell, as both PC1/3 and PC2 have been identified in the rat antrum in high concentrations (Macro *et al.,* 1996).

The N-terminal 1-17 fragment of G34 has a short half life (around 2.5 minutes) and has no known biological activity (Pauwels *et al.,* 1984). The C terminal extensions of progastrin are not effective in stimulating histamine

release (Sandvik and Dockray, 1999) and, therefore, are probably not biologically active.

1.1.5 Secretion, metabolism and blood levels

Gastrin secretion from tissues and subsequent metabolism determine gastrin levels in blood. There are discrepancies between the proportions of the different forms of gastrin in tissue and those in the circulation and reports on the clearance of gastrin have been inconsistent between species. The secretion of G17, G34, and the glycine extended gastrins from the antrum, and the concentrations, proportions and clearance from circulation of these forms of gastrin are discussed in the following sections.

1.1.5.1 Antral secretion of amidated gastrins

The gastrin content of the antra of ten species, including humans, pigs, dogs, cats, rats, mice, rabbits, guinea pigs, sheep and cattle, is approximately 95% G17 and 5% G34 (Andersen, 1985) G34 is slightly higher in foetal lamb tissues (Shulkes and Hardy, 1982b). G34 accounts for a greater percentage of gastrin in blood than in antral tissue in humans (Calam *et al.,* 1980), pigs (Christiansen *et al.,* 1978), dogs (Dockray *et al.,* 1982) and sheep (Simpson *et al.,* 1993). In humans, more G34 is present in the circulation than G17 (Yalow and Berson, 1970; Rehfeld, 1972; Lamers *et al.,* 1982). Lamers *et al.* (1982) proposed that the high G34 content in human blood is due to a large proportion of gastrin originating from the duodenum, which produces mainly G34 (Malmstrom *et al.,* 1976; Calam *et al.,* 1980). In pigs (Simpson *et al.,* 1994) or sheep (Simpson *et al.,* 1993), however, the gastrin content of the duodenum was much less than in the antrum and G17 was generally the major form of gastrin present in the duodenum, although G34 became more prominent in distal portions where gastrin concentration was low.

In dogs, gastrin in the antral vein after feeding consists of 20% G34 and component I, despite a much higher proportion of G17 in antral tissue (Rehfeld and Uvnas-Wallenstein, 1978). Similar proportions were observed in sheep (Simpson *et al.,* 1993). The G34 content in the antral vein is considered to be sufficient to account for the higher proportion of G34 in blood (Rehfeld and Uvnas-Wallenstein, 1978).

Scott *et al.* (1998a) suggested that the increased proportion of G34 in serum may be due to preferential secretion of G34 over G17. Gastrin secretion occurs via secretory granule exocytosis (Mortensen *et al.,* 1979; Oomori *et al.,* 1997) in common with other peptides (Burgess and Kelly, 1987; Rothman and Orci, 1992). Amidated gastrin is secreted by the "regulated secretory pathway," in GH_3 transfected cells at least, and thus secretion is coupled to an extracellular stimulus (Varro *et al.,* 1996). Both G17 and G34 are present in the same population of secretory vesicles, in which G34 is cleaved to G17 (Varro *et al.,* 1993, 1994, 1995, 1996). Gastrin-containing vesicles vary from small immature granules containing progastrin and G34 close to the Golgi complex, to large granules, which contain G17 (Alumets *et al.,* 1979, 1980; Hakanson *et al.,* 1982; Varndell *et al.,* 1983; Rahier *et al.,* 1987), with the electron density of granules dependent upon fixation conditions (Mortensen *et al.,* 1977). Thus, preferential G34 secretion would require preferential release of immature vesicles. Although only one population of vesicles exists, recent research has shown that there are multiple pathways in "regulated secretion" of neuroendocrine cells.

In some endocrine cells, newly synthesised proteins normally part of the regulated secretory pathway, can be released by a "constitutive-like pathway" (Arvan *et al.,* 1991; Kuliawat and Arvan, 1992; Ogawa *et al.,* 1999), although this route involves only a small amount of the total secreted protein, making it an unlikely cause of increased G34 in blood. However, it has been shown that some neuroendocrine cells preferentially release immature vesicles under conditions of low stimulation (Arvan and Castle, 1998). If this were the case for G cells, preferential secretion of G34 could occur, but this is not proven as yet.

The increased concentration of G34 in antral blood may also be partly due to post-secretory enzymatic cleavage of G17. There is evidence of such cleavage as up to 11% of gastrin in the gastroepiploic vein in sheep is G14 (Simpson *et al.,* 1993). Gastrin peptides smaller than G17 account for 30-50% of antral vein immunoreactivity in pigs (Christiansen *et al.,* 1978; Power *et al.,* 1986) and nearly 80% of antral vein immunoreactivity in dogs (Dockray *et al.,* 1982). Endopeptidase 24.11, or an enzyme with similar activity, is present in the antrum, and is at least partly responsible for the post-secretory cleavage of

G17 (Power *et al.,* 1987, 1988; Bunnett *et al.,* 1988; Deschodt-Lanckman *et al.,* 1988), though other enzymes may also exist (Bunnett *et al.,* 1988). Infusion of phosphoramidon, an Endopeptidase 24.11 inhibitor, reduced G17 degradation in the pig antrum, such that G17 accounted for >80% of antral vein gastrin immunoreactivity (Power *et al.,* 1987). Sulphated G17 is more resistant to post-secretory cleavage than unsulphated G17 (Power *et al.,* 1986). Human and porcine G17 are cleaved by Endopeptidase 24.11 at Asp16-Phe17, Gly13-Trp14 and Ala11-Tyr12, with human G17 also cleaved at Trp4-Leu5 and Trp14-Met15 (Bunnett *et al.,* 1988). Of these cleavages, both the Asp16-Phe17 and the Gly13-Trp14 cleavages are rapid with human G17, forming G1-16, G1-13 and G4 (Deschodt-Lanckman *et al.,* 1988). G4 is identical to CCK4, which is biologically active, but is rapidly metabolised in plasma (Koulischer *et al.,* 1982). Whether the products of G17 cleavage could be detected by the gastrin antibodies in studies where increased concentrations of G34 were reported in the antral vein (Uvnas-Wallenstein and Rehfeld., 1978; Simpson *et al.,* 1993) is unknown. It would be expected that G4 and G1-16 would not be detected by an amidated-gastrin-specific antiserum.

1.1.5.2 Antral secretion of glycine-extended gastrins

As for G34, the relative proportion of G-Gly to G17 is increased in blood when compared with tissue. Most results suggest G-Gly account for less than 10% of total tissue gastrin (Hilsted, 1991). When compared with tissues, higher ratios of G-Gly to amidated gastrins are observed in the circulation of pigs (Hilsted *et al.,* 1988a; Hansen *et al.,* 1996a), humans (Hilsted *et al.,* 1988b), sheep (Ciccotosto and Shulkes, 1992) and other species (Hilsted, 1991). There is no evidence of conversion of G17Gly to G17 in the circulation in pigs (Hansen *et al.,* 1996b), perhaps suggesting preferential secretion of G17Gly. Glycine extended gastrins would be present in immature secretory granules as substrates for amidation, and also in mature granules, as G17 and G17Gly occur in the same vesicles (Varro *et al.,* 1995). Glycine extended and amidated gastrins are co-secreted into the antral vein in response to a meal in pigs (Hilsted *et al.,* 1988a) and in response to bombesin from both isolated canine antral G cells (Sugano *et al.,* 1987b) and rat antral tissue (Azuma *et al.,* 1987). A concentration of glycine extended gastrin in general circulation could be due

to preferential secretion of immature vesicles containing more glycine extended gastrin than amidated gastrin, as with G34, or slower post secretory breakdown of glycine extended forms.

In humans, amidated gastrins become more prominent in blood after a meal (Hansen *et al.,* 1996c), possibly due to a change in the amidation levels in the G cells (Varro *et al.,* 1990a, b). When the amidation rate is decreased in rats, glycine extended forms of gastrin in tissue and serum increase, but amidated forms in serum remain the same (Dickinson *et al.,* 1990a). These results suggest that secretion of gastrin from G cells is regulated according to the level of amidated gastrin in the circulation and that G-Gly are co-released with amidated forms.

1.1.5.3 Tissue metabolism of gastrin

When injected into the general circulation, the half-life of human G17 is 4-10 minutes in humans, 3.5 minutes in dogs (Boniface *et al.,* 1976), and even less in cats (Boniface *et al.,* 1976; Rehfeld and Uvnas-Wallenstein, 1978). The half-life of G34 is about five times longer than unsulphated G17 in humans (Walsh *et al.,* 1976), dogs (Walsh *et al.,* 1974) and cats (Rehfeld and Uvnas-Wallenstein, 1978) but 12 times longer than that of human G17 in grower pigs (Xu and Cranwell, 1992). Clearance of the sulphated molecule is three to five times slower than that of the non-sulphated molecule (Pauwels *et al.,* 1987). Of the smaller amidated forms, human G17 and G14 appear to have similar elimination characteristics in dogs (Carter *et al.,* 1979).

Clearance rates for G17 are higher in the foetal lamb (Shulkes and Hardy, 1982a) and the neonatal pig (Xu and Cranwell, 1992) than in older animals, possibly due to increased relative organ weights in young animals. Conversely, clearance rates of G34 are decreased in neonatal pigs, due to decreased G34 clearance by organs (Xu and Cranwell, 1992).

Gastrin clearance across both organs and vascular beds has produced varied results between species. In general, the brain, gut and kidney eliminate G17, as shown in sheep (Ciccotosto and Shulkes, 1992), pigs (Hansen *et al.,* 1996b) and dogs (Becker *et al.,* 1973b; Strunz *et al.,* 1978a). The hindleg was also a significant site of gastrin clearance in studies in dogs (Strunz *et al.,* 1978a) and pigs (Hansen *et al.,* 1996b). More specific studies of the gut in

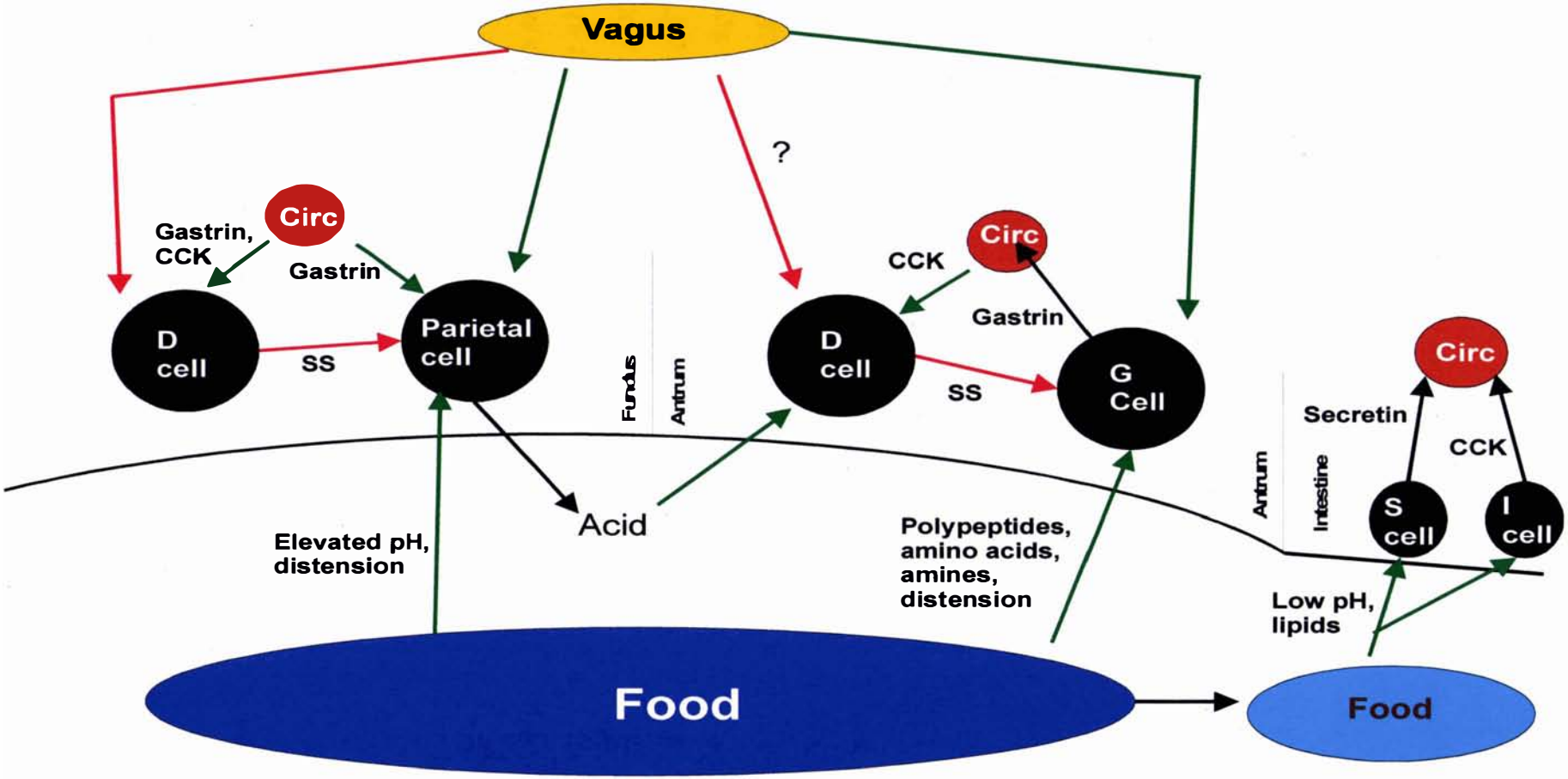

Figure 1.2. Effect of food on gastric secretion. Shown are the cephalic (yellow), gastric (dark blue) and intestinal (light blue) effects of food intake on gastric secretion, with principle effector cells in black. Black arrows indicate release, red arrows an inhibitory effect, and green arrows a stimulatory effect. Abbreviations are explained in the List of Abbreviations, pages XX and XXI.

dogs have shown the fundus to eliminate gastrin (Evans *et al.,* 1974; Alumets *et al.,* 1980). Ciccotosto and Shulkes (1992) reported the lungs to be the most important site of gastrin clearance in sheep, but this was not the case in pigs (Hansen *et al.,* 1996b).

Clearance of gastrin across the liver has been frequently shown to vary between species. In rats (Doyle *et al.,* 1984) and dogs (Strunz *et al.,* 1978b), the liver removed most fragments of less than eight amino acids long in the first pass, but 90-95% of larger fragments pass through intact (Doyle *et al.,* 1984). Thus, any peptides smaller than eight amino acids are unlikely to have any physiological function. In pigs, 50% of whole body elimination of amidated gastrin occurs across the liver (Christiansen *et al.,* 1982; Hansen *et al.,* 1996b), with G14 almost completely removed (Christiansen *et al.,* 1978) and G17 and G34 also partially metabolised (Christiansen, 1984; Xu and Cranwell, 1992). Removal of gastrin by the liver is proportional to its circulating concentration, obeying first order kinetics, thus endocrine responses are still possible (Christiansen *et al.,* 1982; Hansen *et al.,* 1996b). Hansen *et al.* (1996a, b) suggested disparity between studies on gastrin clearance by the liver may be due to the form of G17 used.

The clearance rate of G17 and G17Gly is similar in sheep (Ciccotosto and Shulkes, 1992), pigs (Hansen *et al.,* 1996b) and humans (Hilsted *et al.,* 1988b), with a half-life of 4-22 minutes in humans (Hansen *et al.,* 1996c). Clearance sites for G17Gly and G17 are the same in sheep, but G17Gly is not removed by the liver or kidney in pigs (Hansen *et al.,* 1996a, 1999). The kidney eliminates NH_2-terminal progastrin as well as amidated gastrin (Ciccotosto *et al.,* 1996; Hansen *et al.,* 1999).

1.1.6 Integrated physiological control of gastrin release

Regulation of gastrin secretion in monogastric animals principally revolves around feeding and the presence of food in the gastrointestinal tract. Gastrin secretion has classically been divided into a cephalic, a gastric and an intestinal phase. The mechanisms through which gastrin secretion is controlled involve neural, hormonal and chemical components (outlined in Figure 1.2).

1.1.6.1 The cephalic phase

The cephalic phase of gastrin secretion involves neural pathways activated by the sight, smell and taste of food and is transmitted to the stomach by the vagus nerve (reviewed by Dockray and Gregory, 1989).

The effects of the vagus nerve on gastrin secretion have been studied extensively, using selective and truncal vagotomy. Such procedures have led to a confusing diversity of results. Probably the most serious complication in these studies has been the failure to control gastric pH, as vagotomy removes vagal stimulation of the parietal cell (Stadil, 1972), elevating gastric pH, which in turn stimulates gastrin release. In fact, in one experiment in rats, gastrin secretion was unaffected by vagotomy except when acid secretion was decreased (Magellanes *et al.,* 1982). A further complexity in vagotomy experiments is how soon after the vagotomy the experiments were conducted, as response patterns change within a week of the operation (Hollinshead *et al.,* 1985).

There are vagal pathways which stimulate gastrin release (Hirschowitz *et al.,* 1979; Alumets *et al.,* 1980), via Gastrin Releasing Peptide (GRP)-ergic and cholinergic mechanisms (Weigert *et al.,* 1993). The cephalic phase of gastrin secretion involves vagal stimulation of gastrin release, shown in dogs in response to feeding (Dockray and Tracy, 1980b) and sham feeding (Hirschowitz and Fong, 1990), and occurs via stimulation of the non-cholinergic (probably GRPergic) pathway (Dockray and Tracy, 1980a, b).

There are also inhibitory vagal pathways that inhibit gastrin release which cannot be explained by acid secretion (Debas *et al.,* 1976, Debas and Carvajal, 1994; Hughes and Hernandez, 1976; Feldman *et al.,* 1979; Hirschowitz *et al.,* 1979). It is unclear when these reflexes are important, but they do not appear to be involved in the cephalic phase of gastrin secretion.

1.1.6.1.1 The cephalic phase in ruminants

The gastrin response to feeding may be reduced or absent in ruminants on a high level of food intake (Reynolds, 1982; Perry *et al.,* 1988; Reynolds *et al.,* 1991). Thus, the cephalic phase of gastrin secretion is unlikely to be obvious in a pasture or *ad libitum* fed ruminant and may even be diminished. However, sheep on restricted feeding regimens exhibit more extreme

fluctuations in gastrin secretion, with peak responses coinciding with feeding times (Reynolds, 1982; Lawton *et al.,* 1996). These gastrin responses to feeding are likely to be similar to abomasal secretion and have a cephalic component (McLeay and Titchen, 1970). Stimulation of the anterior and posterior vagal trunks in sheep increased serum gastrin levels (Bladin *et al.,* 1983), though the changes observed were minute. Gastrin release has also been reported in response to vagal stimulation in calves (Adrian *et al.,* 1983). These results imply that vagal control pathways do exist in sheep, but may not be prominent in ruminants on pasture which are feeding *ad libitum*.

1.1.6.2 The gastric phase

The gastric phase of gastrin secretion has traditionally been regarded as involving both neural and chemical factors. However, many of the luminal chemical factors, such as acid and polypeptides, have been shown to act through neural pathways and thus may not stimulate the G and D cells directly.

The presence of food in the lumen in contact with the gastric mucosa appears necessary to maintain gastrin production and secretion. The passage of food through the antrum is necessary for G cell hyperplasia in fundectomised rats (Alumets *et al.,* 1980). Studies in rats showed fasting for 48 hours (Track *et al.,* 1978) or four days (Lichtenberger *et al.,* 1975) markedly reduced serum and tissue gastrin concentrations. Similar results are also reported for rats fed a non-nutritious diet, suggesting certain chemical constituents of the diet are necessary for maintenance of gastrin levels (Lichtenberger *et al.,* 1975).

1.1.6.2.1 Proteins, polypeptides and amino acids

Foods containing amino acids are potent stimulants of gastrin release, whereas fats and carbohydrates are not (Richardson *et al.,* 1976). In humans, gastric instillation of an amino acid solution, but not an albumin solution, stimulated gastrin and acid secretion (Richardson *et al.,* 1976), with observed increases in circulating gastrin levels sufficient to account for all of the acid secretion stimulated by amino acids (Feldman *et al.,* 1978). Blood gastrin concentrations increase in response to a peptone meal in dogs (Dockray *et al.,* 1980b; Varner *et al.,* 1981; Fung and Greenberg, 1997), humans (Konturek *et al.,* 1974; Walsh *et al.,* 1976), in the isolated perfused rat stomach (Saffouri *et*

al., 1984b; Schubert *et al.,* 1992) and with casein hydrolysate irrigation of antral pouches in dogs (Gabrys-Pomykala *et al.,* 1977). In sheep, gastric secretion is increased by intra-abomasal infusion of products of protein digestion (van Bruchem and van T'Klooster, 1980). A mixture of polypeptides and amino acids is more potent in stimulating gastrin release than each of these on its own (Walsh *et al.,* 1976).

Stimulation of gastrin release by polypeptides is mediated by intramural reflexes and can be abolished by tetrodotoxin (Schubert *et al.,* 1992). Similarly, in rats, the acid response to peptone infusion, mediated by gastrin, is inhibited by capsaicin (Ramos *et al.,* 1992).

In contrast to polypeptides, amino acids are thought to act directly on the G cell to stimulate gastrin release. The effects of amino acids are thought to be receptor mediated (DelValle and Yamada, 1990). It has been reported that in humans, cholinergic nerves stimulate gastrin release in response to amino acid meals (Schiller *et al.,* 1982), though this effect may be due to the presence of peptides. Hydrophobic amino acids, particularly tryptophan and phenylalanine, seem to be most effective in stimulating gastrin release in dogs (Konturek *et al.,* 1977a), humans (Taylor *et al.,* 1982) and rats (Lichtenberger *et al.,* 1982b; Dial *et al.,* 1991). Amino acid stimulation of gastrin release, like that by polypeptides, is dependent upon gastric acidity and is reduced at low gastric pH (Walsh *et al.,* 1976).

Dietary amines also stimulate gastrin release (Lichtenberger *et al.,* 1982a) via a non-specific, non-receptor action (DelValle and Yamada, 1990). Amines stimulate gastrin release by diffusing into the G cell and alkalinise the interior of gastrin secretory granules, stimulating exocytosis (DelValle and Yamada, 1990; Dial *et al.,* 1991). While decarboxylation of amino acids enhances their ability to stimulate gastrin secretion (Lichtenberger *et al.,* 1982b; Dial *et al.,* 1986, 1991), amino acids do not stimulate gastrin release by being converted to amines. Inhibition of endocrine cell decarboxylases, which convert amino acids to amines, did not affect the stimulation of gastrin by amino acids (DelValle and Yamada, 1990). However, inhibition of monoamine oxidase, which metabolises amines, does enhance meal-stimulated gastrin release (Dial *et al.,* 1986). Thus, amines in food do stimulate gastrin release but amino acids

are not converted to amines by tissue. McIntosh *et al.* (1984) reported that cystamine-induced gastrin secretion could be reduced by Gastric Inhibitory Polypeptide (GIP)-induced somatostatin, but DelValle and Yamada (1990) reported somatostatin had no effect. Thus amines and amino acids seem to stimulate gastrin release by different mechanisms.

1.1.6.2.2 Gastric Acidity

Acidity of the stomach or abomasum plays a central role in controlling gastrin release. Gastrin secretion and gastric acidity are reciprocally linked, with the fundamental action of gastrin being regulation of acid secretion.

There is less buffering of juxtamucosal pH by mucus in the antrum than in the fundus (Quigley and Turnberg, 1987). This may facilitate antral, and therefore G cell sensitivity, to gastric pH. Elevation of antral juxtamucosal pH has been proposed as the cause of hypergastrinaemia in *H. pylori* positive patients (Kelly *et al.,* 1993).

Increased gastric pH increases blood gastrin levels. This has been shown in experiments in which antral pH has been elevated by infusion of high pH solutions (Becker *et al.,* 1973a; Smith *et al.,* 1975), fundectomy (Alumets *et al.,* 1979, 1980; Hakanson *et al.,* 1982), antral exclusion (Alumets *et al.,* 1980; Varro *et al.,* 1990a), or inhibition of acid release by omeprazole (Allen *et al.,* 1986; Brand and Fuller, 1988; Larsson *et al.,* 1988; Dimaline *et al.,* 1991; Dockray *et al.,* 1991, 1993; Takehara *et al.,* 1996). Elevation of gastric pH also increases the gastrin response to a meal (Dockray and Tracy, 1980a). Conversely, lowering gastric pH reverses the elevation in gastrin secretion in response to hypoacidity (Becker *et al.,* 1973a; Dockray *et al.,* 1993) and decreases gastrin release in response to neural stimulation (Hirschowitz *et al.,* 1979) and a meal (Konturek *et al.,* 1995). Similarly, gastric acid secretion decreases as antral pH decreases (Antinone *et al.,* 1967) an effect probably mediated by gastrin. Omeprazole infusion is also effective in reducing acid secretion and increasing blood gastrin concentrations in sheep (Shulkes and Hardy, 1982a). Syndromes reducing acid secretion also tend to involve a hypergastrinaemia as in 5 and 10 week old obese mice (Morton *et al.,* 1985). Most evidence therefore suggests that increased gastric pH increases gastrin

secretion from the antrum, and that low gastric pH reduces the sensitivity of the G cell to stimulation.

There are reports from studies in humans that do not support stimulation of gastrin secretion due to increased gastric pH alone. These studies suggest that other stimulants are necessary to increase gastrin secretion in response to high gastric pH (Arnold *et al.,* 1984). No increase in blood gastrin concentration was observed in studies where gastric pH was elevated using H_2 receptor antagonists (Mohammed *et al.,* 1983; Arnold *et al.,* 1984; Fabri *et al.,* 1989), antacids (Arnold *et al.,* 1984) or omeprazole (Fabri *et al.,* 1989) in humans. Furthermore, it has been proposed that a fundic inhibitor which is a neural mechanism, not acid secretion, is responsible for inhibition of gastrin secretion (Penston and Wormsley, 1987; Magee, 1996). In general, however, acid secretion is accepted as a central component in the control of antral gastrin secretion.

Somatostatin has been shown to be the inhibitory effector through which luminal acidity controls gastrin levels. Decreasing gastric pH is associated with an increase in somatostatin release in the perfused mouse stomach (Schubert *et al.,* 1988) and in pigs (Holst *et al.,* 1983). In mice (Schubert *et al.,* 1988) and dogs (Greenberg *et al.,* 1993), the effect of luminal acidity is directly on the D cell (Schubert *et al.,* 1988). In rats, however, the effects of gastric acidity on gastrin secretion are mediated by cholinergic and noncholinergic neurons (Saffouri *et al.,* 1984b). The increased gastrin secretion in rats in response to omeprazole-induced hypoacidity is stimulated by GRP neurons (Takehara *et al.,* 1996), while the somatostatin response to acidity is mediated by Calcitonin Gene Related Peptide (CGRP) containing neurons (Manela *et al.,* 1995; Ren *et al.,* 1992).

1.1.6.2.3 Ionic effects

In humans, magnesium, aluminium (Peterson *et al.,* 1986a) and calcium (Behar *et al.,* 1977) stimulated gastrin release. In dogs, however, calcium, magnesium and sodium ions stimulated acid, but not gastrin, secretion (McLaughlin *et al.,* 1978). In cats, phosphate, bicarbonate and hydroxide ions stimulated gastrin secretion (Uvnas-Wallenstein, 1978), possibly due to buffering effects. The variation in results observed may be due to species

differences, contact time and concentration of ions. Gastrin cells contain calcium sensing receptors which stimulate gastrin, so luminal ions may be able to affect gastrin secretion (Ray *et al.,* 1997a; Squires *et al.,* 1999).

1.1.6.2.4 Gastric distension

Distension has been shown to stimulate both acid and gastrin secretion. However, studies have produced a wide range of results, both within and between species, possibly due to different degrees of distension and different neural pathways between species.

In dogs, gastrin secretion is increased more by distension of vagally innervated isolated antral pouches (Gabrys-Pomkala *et al.,* 1977) than by denervated pouches (Konturek *et al.,* 1977a), suggesting the presence of distension-sensing neurons in the antrum. Such neurons are probably also present in the fundus, as distension of the fundic region in dogs also stimulated gastrin release (Debas *et al.,* 1975).

In humans, distension of the stomach may increase acid secretion via pathways not involving gastrin. Distension in humans stimulates acid secretion (Richardson *et al.,* 1976; Soares *et al.,* 1977), but either none (Richardson *et al.,* 1976) or only small amounts of gastrin are released and only at high pressures (Soares *et al.,* 1977; Schiller *et al.,* 1980; Peters *et al.,* 1982). Gastrin induced distension is not via cholinergic mechanisms (Schiller *et al.,* 1980; Peters *et al.,* 1982) but via a β-adrenergic mechanism (Peters *et al.,* 1982).

Results from rats are the most contradictory. Schubert and Makhlouf (1993) reported that low grade distension inhibited gastrin release via somatostatin, while increased distension stimulated gastrin via cholinergic neurons in the perfused rat stomach. In contrast, Weigert *et al.* (1997) reported that intrinsic GRP neurons were responsible for stimulatory effects of distension *in vivo* in rats, while in the isolated rat stomach distension inhibited gastrin secretion via cholinergic and other unknown mechanisms. Higham *et al.* (1997) reported almost opposite effects to those of Weigert *et al.* (1997), which suggested that antral innervation inhibits G cell responses to distension. These different results may be due to differing degrees and methods of denervation, or differing levels of distension.

1.1.6.3 Intestinal phase

The intestinal phase is mediated by hormonal feedback, stimulated by acidity and the components of the digesta in the duodenum. During the intestinal phase both gastrin and acid secretion are reduced. Many of the intestinal hormones affecting gastric secretion, enterogastrones, appear to be more effective on acid secretion than on gastrin.

Duodenal acidity has an inhibitory feedback on both gastrin and acid secretion from the stomach, possibly via secretin (Konturek *et al.,* 1977b). In addition, infusion of intraduodenal fat, but not casein or glucose, caused a prompt increase in antral somatostatin release in dogs, an effect suggested to be mediated by either secretin or cholecystokinin (Schudziarra *et al.,* 1978). Although secretin (Konturek *et al.,* 1977b) and cholecystokinin (Konturek *et al.,* 1995) are released by high acidity in the duodenum, the actions of both on gastrin secretion are debatable. There is evidence both for (Chey *et al.,* 1981; Kim *et al.,* 1981) and against (Kleibeuker *et al.,* 1984; You and Chey, 1987) the inhibition of gastrin by secretin at physiological levels. However, secretin can inhibit acid secretion, even if it does not affect gastrin secretion (Sandvik *et al.,* 1987; You and Chey, 1987). In humans, cholecystokinin inhibits gastric acid secretion and sometimes also gastrin (Konturek and Konturek, 1993; Konturek *et al.,* 1993). CCK_A receptor antagonists increase blood gastrin concentration in response to various stimuli (Beglinger *et al.,* 1992; Katschinski *et al.,* 1992; Konturek *et al.,* 1992; Schmidt *et al.,* 1994). However, Lloyd *et al.* (1992) noted intestinal lipids and CCK_A blockade had no effect on gastrin release stimulated by a peptone meal, but inhibited acid secretion. Konturek (1992) proposed that these results could have been due to an inadequate dose of the receptor antagonist. In rats, however, CCK_A receptor blockade did not affect basal or meal stimulated acid secretion (Varga *et al.,* 1993).

Other hormones released by the intestine also affect gastric secretion. Neurotensin, Peptide YY (PYY) and Glucagon Like Peptide 1 (GLP1) are considered enterogastrones, as they are intestinal hormones which inhibit acid secretion (Schubert, 1998). While both PYY and GLP1 inhibit acid secretion, neither affects gastrin secretion (Lloyd *et al.,* 1997a; Fung *et al.,* 1998). Therefore, enterogastrones may not affect acid secretion via gastrin.

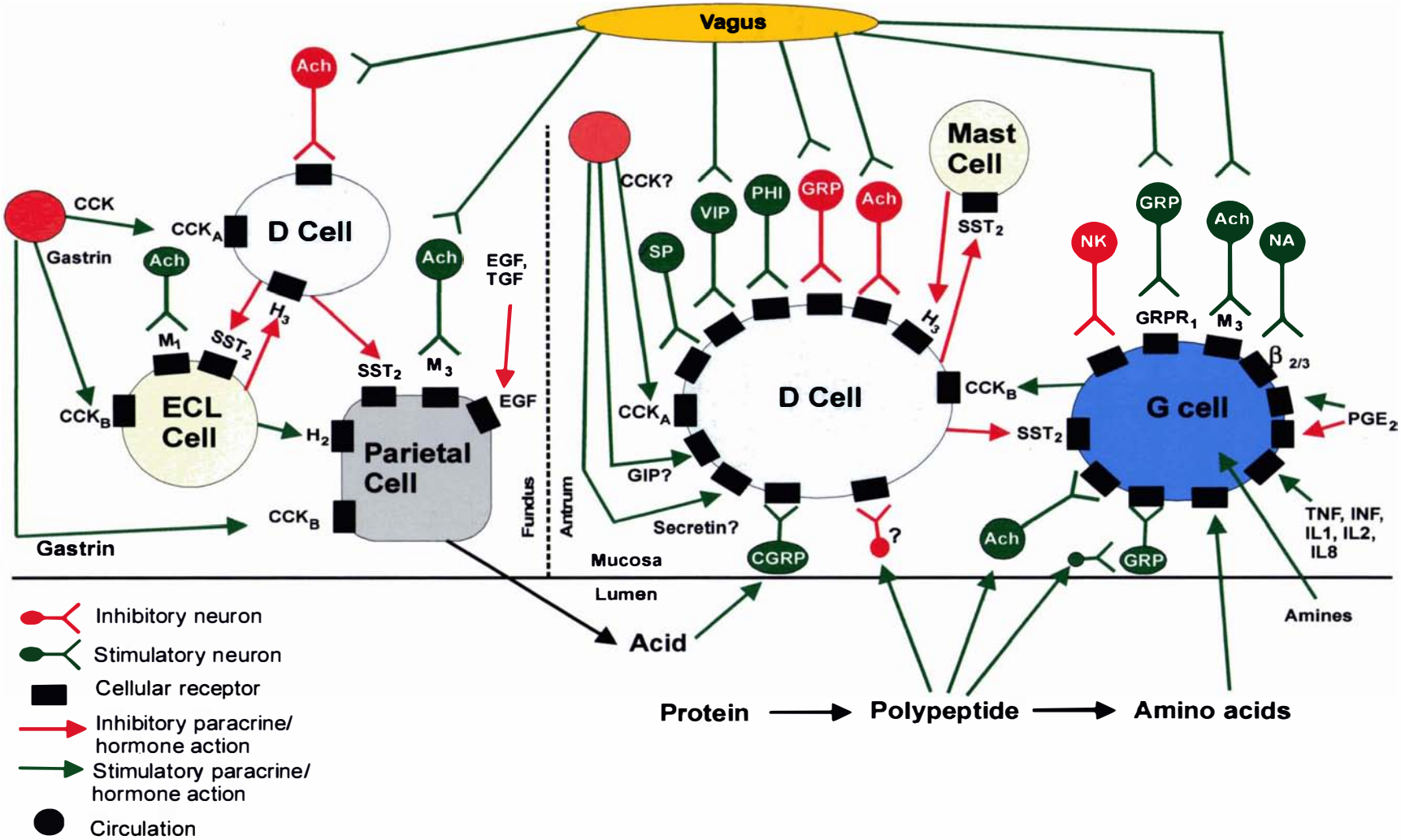

Figure 1.3. Cellular control of gastrin and acid secretion. Shown are the factors controlling gastrin and acid secretion, with emphasis on the control of gastrin secretion. All abbreviations are explained in the List of Abbreviations, pages XX and XXI.

1.1.7 Cellular control of gastrin secretion

The principal cellular mechanisms controlling gastrin secretion are shown in Figure 1.3. At the cellular level, gastrin secretion by the antral G cell is regulated by effector molecules acting either directly on the G cell, or by reducing somatostatin release from the antral D cell, which inhibits gastrin release. Secretion from both G cells and D cells is regulated by neurotransmitters from the local intramural network, notably GRP, acetylcholine, Vasoactive Intestinal Polypeptide (VIP), noradrenaline and CGRP, and possibly others, such as the tachykinins, γ-amino butyric acid (GABA), adenosine and Peptide Histidine Isoleucine (PHI). Antral G and D cells may be also regulated by hormones such as secretin and cholecystokinin and possibly also insulin and catecholamines. Finally, the G and D cells are influenced by paracrine mechanisms such as inflammatory mediators, which are discussed in detail in Section 1.2.1.2.

1.1.7.1 Somatostatin and inhibition of gastrin secretion

Antral somatostatin is the primary inhibitor of antral gastrin secretion. The D cell is the integration point at which many of the factors that inhibit gastrin release exert their effect. Some inhibitors of gastrin secretion may also act directly on the G cell, though reports of such effects are rare.

Somatostatin inhibits gastrin release by direct action on the G cell (Campos *et al.,* 1990). The somatostatin receptor subtype SST_2 is responsible for inhibiting gastrin release in humans, dogs and rats (Zaki *et al.,* 1996; Fung and Greenberg, 1997; Lloyd *et al.,* 1997b). The SST_2 receptor has three separate isoforms, all with the same pharmacological actions, but of which one form predominates in gastrin control (Sandvik *et al.,* 1995). Harty *et al.* (1985) also noted post-receptor actions of somatostatin and suggested that it may be internalised in a similar fashion to insulin.

Saffouri *et al.* (1979, 1980) first reported that somatostatin exerted a continuous restraint on basal gastrin secretion. Somatostatin also alters gastrin mRNA levels, and thus affects gastrin production as well as secretion (Karnik *et al.,* 1989). Immunoblockade of somatostatin stimulates gastrin release in pigs (Holst *et al.,* 1992), perfused rat stomachs (Saffouri *et al.,* 1979; Short *et al.,*

1985a) and rat antral tissue (Chiba *et al.,* 1981; Wolfe *et al.,* 1986; Zaki *et al.,* 1996), humans (Zaki *et al.,* 1996) and dogs (Zaki *et al.,* 1996).

Somatostatin is believed to have a maximal level of restraint on gastrin secretion (Saffouri *et al.,* 1984a). The basal level of somatostatin restraint on gastrin secretion varies between species. Somatostatin inhibited basal gastrin secretion from human, rat and canine antral mucosal tissue fragments (Zaki *et al.,* 1996), and porcine antral mucosa (Holst *et al.,* 1992) inhibited basal gastrin secretion. Thus, basal restraint of gastrin by somatostatin is not maximal in these species. However, other studies using human antral G cells (Campos *et al.,* 1990) and rat antral mucosa (Hayes *et al.* 1975) somatostatin did not affect basal gastrin secretion. Likewise, Lawton (1995) reported that increased somatostatin secretion induced by VIP and GIP did not affect basal gastrin secretion by sheep antral fragments. These variable results may be an effect of differing tissue preparations.

In rats, humans and baboons, somatostatin-secreting antral D cells are located adjacent, or close to, G cells (Larsson *et al.,* 1979a; Buchan *et al.,* 1985) and have basal extensions which are present close to the basal portions of the G cell (Larsson *et al.,* 1979a; Buchan *et al.,* 1985; Polak, 1986). Some D cells also make contact with the lumen (Polak, 1986; Holst *et al.,* 1993) and may thus be under direct control from dietary constituents.

The effect of somatostatin on gastrin secretion is generally accepted to be paracrine. This is supported by: the close proximity of D cells to G cells; the inability to inhibit basal or meal stimulated gastrin secretion by infusion of somatostatin to produce postprandial peripheral concentrations (Colturi *et al.,* 1984; Fung and Greenberg, 1997); and in some studies, the necessity for somatostatin antiserum to be present at the site of secretion to create an effective blockade of activity (Chiba *et al.,* 1981; McIntosh, *et al.,* 1991). Furthermore, studies in sheep show peripheral somatostatin concentration is much lower than antral or fundic concentrations. Even after stimulation of somatostatin release using either gastrin or cholecystokinin, jugular vein concentrations of somatostatin were half those present in the antral vein prior to stimulation (Zavros *et al.,* 1998). Thus, circulating somatostatin levels, which are much lower than antral tissue levels, are unlikely to affect gastrin secretion.

That the effect of somatostatin on gastrin secretion is paracrine is important, as the fundus also contains D cells, which have been widely reported to inhibit acid release (reviewed by Dockray and Gregory, 1989; Schubert, 1994, 1997, 1998). Antral and fundic somatostatin release are controlled differently (Schubert *et al.,* 1991; Zavros *et al.,* 1998, 1999). This suggests that somatostatin from the antrum and the fundus have different functions, antral somatostatin controlling gastrin secretion and fundic somatostatin controlling acid secretion. In sheep, more somatostatin is released from the fundus than from the antrum, and peripheral somatostatin best reflects fundic somatostatin secretion (Zavros *et al.,* 1998).

Somatostatin infusion *in vivo* has produced varied results, which may be due to its inhibition of both the G cell and acid secretion, producing competing effects on gastrin secretion. Intravenous infusion of somatostatin has been variously reported to decrease circulating gastrin in rats (Kondo *et al.,* 1993) and sheep (Shulkes and Hardy, 1982a), to have no effect in dogs (Gomez *et al.,* 1997) or to increase gastrin secretion in sheep (Barry *et al.,* 1985; Reynolds *et al.,* 1991).

The role of somatostatin in restraining both gastrin and acid secretion and in the regulation of gastrin by acidity, has been questioned. Westbrook *et al.* (1998) reported that chronic immunisation of sheep against somatostatin affected neither gastrin nor acid secretion. The suitability of chronic immunisation in the assessment of the paracrine actions of somatostatin was questioned by Schubert (1998). Further, the partitioning of fundic and antral somatostatin into separate paracrine effects was questioned by Zavros *et al.* (1999). These authors reported that fundic, not antral somatostatin release was responsible the inhibition of a gastrin response to GRP in sheep *in vivo.* It was proposed that this action may be via an endocrine or a local fundic-antral portal connection (Zavros *et al.,* 1999). It is possible that these results were influenced by anaesthesia of the subject animals, which markedly stimulated basal somatostatin secretion. Examination of the results obtained also showed abomasal pH was increased during experiments, also due to anaesthesia (Reynolds GW and Carr DH, unpublished results), which may in turn have affected the responses obtained.

1.1.7.1.1 Control of somatostatin secretion

Somatostatin release, and subsequent inhibition of gastrin secretion, is stimulated by hormones, particularly of intestinal origin, and neuropeptides present in the intramural neurons of the antrum. Many peptides have been shown to affect gastrin and somatostatin secretion. The stimulants of somatostatin secretion, discussed below, are released in response to vagal stimulation or gastric acidity, and thus may have a physiological role in regulating antral somatostatin and gastrin secretion. Other neurotransmitters, such as adenosine (Kwok *et al.,* 1990; Schepp *et al.,* 1990), have been shown to stimulate gastrin and somatostatin secretion, but their biological role is unknown. In addition to neural and hormonal regulation, antral somatostatin secretion is regulated by feedback loops with gastrin and histamine. These feedback loops occur as local histamine and gastrin concentrations regulate the secretion of somatostatin, while somatostatin in turn regulates the secretion of both local histamine and gastrin.

1.1.7.1.1.1 Vasoactive Intestinal Polypeptide (VIP)

VIP is released by vagal stimulation in pigs, increasing somatostatin secretion via a direct action, and may also inhibit gastrin secretion by a direct action (Holst *et al.,* 1993). Further, in rats, increased VIP levels correlated with increased somatostatin and decreased gastrin at low levels of distension in the rat stomach (Schubert and Makhlouf, 1993). Thus, VIP may be an important neuropeptide in the regulation of antral somatostatin secretion.

VIP is a neuropeptide located throughout the gut (Larsson *et al.,* 1979), including the abomasum in calves (Bloom *et al.,* 1979) and sheep (Reid *et al.,* 1988) and the pig stomach (Holst *et al.,* 1992). In rats, VIP immunostaining is evident in peptidergic nerves in all layers of the stomach wall and in neuronal cell bodies (Allen *et al.,* 1986).

Most evidence suggests that VIP decreases gastrin secretion by stimulating somatostatin release. In the perfused rat stomach, VIP infusion at 10^{-8}-10^{-6}M increased somatostatin and decreased gastrin secretion (Chiba *et al.,* 1980a), but at more physiological doses only increased somatostatin secretion (Saffouri *et al.,* 1984a).

1.1.7.1.1.2 Peptide histidine isoleucine (PHI)

In pigs, vagal stimulation releases both PHI and VIP, and both peptides have been shown to stimulate antral somatostatin release (Holst *et al.,* 1993). PHI is a peptide of the secretin/GIP family (Wolfe, 1989) and is also present in peptidergic nerves in all layers if the stomach wall and in neuronal cell bodies (Allen *et al.,* 1986). In rat antral tissue, 10^{-9}-10^{-7}M PHI inhibited both gastrin secretion and mRNA levels, but these effects were only consistent in the presence of carbachol (Wolfe, 1989).

1.1.7.1.1.3 Tachykinins

In pigs, tachykinins are released during vagal stimulation (Holst *et al.,* 1993). The tachykinin peptides Neuropeptide Y (NY) and Substance P (SP) are observed in neurons in all layers of the stomach wall in rats (Allen *et al.,* 1986), with tachykininergic nerves observed close to G cells in pigs (Schmidt *et al.,* 1996). Various tachykinin peptides have been shown to inhibit gastrin and stimulate somatostatin secretion in rats (Kwok *et al.,* 1985, 1988; McIntosh *et al.,* 1987), dogs (Campos *et al.,* 1989) and pigs (Holst *et al.,* 1987a, 1993; Schmidt *et al.,* 1996).

SP is reported to oppose the action of GRP. SP decreased GRP- and vagal-stimulated gastrin secretion in the isolated pig stomach (Holst *et al.,* 1987a) and modestly decreased bombesin-stimulated gastrin release from canine G cells (Campos *et al.,* 1989). SP stimulated basal canine G cell response (Campos *et al.,* 1989) but did not affect serum gastrin in dogs when infused (Modlin *et al.,* 1981). Holst et al (1993) postulated that tachykinins stimulate somatostatin release from the antrum in pigs, and may also inhibit gastrin release by a direct action. In pig antrum both Neurokinin A and SP inhibit gastrin directly as well as stimulating somatostatin release, though SP is more potent in stimulating somatostatin (Schmidt *et al.,* 1996). The actions of SP on somatostatin are not nicotinic, muscarinic, opiate (Kwok *et al.,* 1985) or histamine (H_1 and H_2) mediated (McIntosh *et al.,* 1987), but are thought to be via substance P-K or neurokinin-2 receptors (Kwok *et al.,* 1988).

1.1.7.1.1.4 Calcitonin gene related peptide (CGRP)

It has been proposed that CGRP neurons regulate somatostatin secretion in response to gastric acidity. In rats, stimulation of somatostatin release by antral acidity is via CGRP neuron stimulation of the D cell (Ren *et al.,* 1992; Manela *et al.,* 1995). D cells in a rat gastric endocrine cell culture respond to CGRP (Zeng *et al.,* 1996), which also modulates somatostatin mRNA expression (Ren *et al.,* 1998). CGRP is abundant in the pig antrum, but has little effect on somatostatin secretion (Holst *et al.,* 1993). Thus, CGRP may not mediate acid stimulation of somatostatin release in all species.

1.1.7.1.1.5 Intestinal hormones

The enterogastrones cholecystokinin, secretin and GIP may inhibit gastrin secretion. These hormones all act by stimulating somatostatin secretion (secretin: Chiba *et al.,* 1980b; Saffouri *et al.,* 1984a; Buchan *et al.,* 1993; GIP: Holst *et al.,* 1983; McIntosh *et al.,* 1984; Wolfe *et al.,* 1986; cholecystokinin: Buchan *et al.,* 1990; 1993; Konturek *et al.,* 1993, 1995; Schmidt *et al.,* 1994; Zavros and Shulkes, 1997; Zavros *et al.,* 1998; Zeng *et al.,* 1996) and in many studies also inhibit gastrin secretion. Somatostatin stimulation and gastrin inhibition are linked in some studies (secretin: Wolfe *et al.,* 1983; GIP: Wolfe *et al.,* 1986; cholecystokinin: Zavros and Shulkes, 1997). Secretin may also have direct effects on the G cell, but this is observed only when high concentrations are used (Saffouri *et al.,* 1984a).

1.1.7.1.1.6 Feedback loops: gastrin and somatostatin

Gastrin itself may be a stimulus to somatostatin release and thus form a feedback loop, inhibiting further gastrin release. Gastrin stimulated somatostatin release in the rat antrum (Schubert *et al.,* 1991) and from dispersed endocrine cells (Zeng *et al.,* 1996), including antral and fundic D cells which have CCK_B receptors in rats (Song *et al.,* 1996) and dogs (Helander *et al.,* 1997). In sheep, G17 stimulated antral, fundic (Zavros *et al.,* 1998) and peripheral (Zavros and Shulkes, 1997; Zavros *et al.,* 1998) somatostatin levels. Inhibition of acid secretion by omeprazole also increased antral (Read *et al.,* 1992), but not peripheral (Shulkes and Read, 1991; Read *et al.,* 1992; Westbrook *et al.,* 1998) somatostatin levels in sheep, possibly due to increased

gastrin secretion. Zavros *et al.* (1998) reported that while gastrin was able to stimulate antral somatostatin secretion this effect could not be reversed by CCK_B receptor blockade. An alternative mechanism by which gastrin could stimulate antral somatostatin secretion was not clear, and it was suggested that a low concentration of CCK_B receptors and a high concentration of gastrin may have rendered the CCK_B receptor antagonist ineffective.

Gastrin does not stimulate antral somatostatin release in all species. In pigs, gastrin does not stimulate somatostatin secretion (Holst *et al.,* 1987b), and vagal stimulation of gastrin is not enhanced by somatostatin immunoblockade (Holst *et al.,* 1992). Instead, somatostatin seems to be stimulated by an autocrine control loop (Holst *et al.,* 1993), luminal acid (Holst *et al.,* 1983), and have neural and hormonal control (Holst *et al.,* 1993). Gastrin was similarly unable to stimulate somatostatin release from human antral D cells (Buchan *et al.,* 1990).

1.1.7.1.1.7 Feedback loops: histamine and somatostatin

Histamine secretion has been suggested to form a negative feedback loop with somatostatin secretion, which amplifies any changes in somatostatin secretion. Histamine inhibits somatostatin release in both the antrum and the fundus (Schubert and Makhlouf, 1996) via the H_3 receptor (Bado *et al.,* 1994; Vuyyuru *et al.,* 1995, 1997). A dual negative inhibitory loop, where histamine inhibits somatostatin secretion and somatostatin inhibits somatostatin secretion, exists in human, dog and rat antral tissue (Vuyyuru *et al.,* 1995) and in the fundus (Vuyyuru *et al.,* 1997). These authors suggest that the dual inhibitory pathway acts to amplify changes in somatostatin secretion (Vuyyuru *et al.,* 1995 and 1997). In sheep, however, Grabau *et al.* (1999) reported that histamine stimulated somatostatin release, an effect that was inhibited by ranitidine, but unaffected by omeprazole. As this experiment was conducted *in vivo* and somatostatin was measured in peripheral circulation, the source of somatostatin and the exact site of histamine action are unclear.

1.1.7.2 Stimulants of gastrin secretion

Stimulation of gastrin release is principally via neural mechanisms. Extensive studies have shown that GRP and acetylcholine stimulate gastrin

secretion by direct, receptor mediated actions on the G cell. Adrenergic receptors have also been demonstrated on G cells and these may also play a role in stimulating gastrin release. There is also stimulation of the G cell via chemical mechanisms, notably amino acids (discussed in 1.1.6.2.1) and inflammatory mediators (discussed in 1.2.3.2).

1.1.7.2.1 Gastrin Releasing Peptide (GRP) /Bombesin

GRP/bombesin is the main neuronal stimulant of gastrin secretion. Bombesin-like immunoreactivity is rich in the stomach of rats (Dockray *et al.,* 1979), in the antral glands of rats and guinea pigs where G, D and Enterochromaffrin (EC) cells are located (Buffa *et al.,* 1982), in antral mucosal, submucosal and myenteric nerve fibres (Holst *et al.,* 1987a) and in nerves close to D cells (Holst *et al.,* 1987b) in pigs.

Gastrin release by electrical vagal stimulation is atropine-resistant in dogs (Smith *et al.,* 1975), rats (Nishi *et al.,* 1987) and pigs (Holst *et al.,* 1987b) and in response to sham feeding in dogs (Dockray and Tracy, 1980a, b). In dogs, bombesin produced similar gastrin stimulatory effects to vagal stimulation (Hirschowitz *et al.,* 1979). In pigs, GRP released by vagal stimulation caused a dose dependent, atropine-resistant increase in gastrin secretion from the perfused stomach or antrum (Holst *et al.,* 1987a, b, 1993). Field stimulation of gastrin release is primarily via GRP in the perfused rat stomach (Schubert *et al.,* 1985), with vagal stimulation at 10Hz releasing gastrin by GRPergic mechanisms (Weigert *et al.,* 1993). Vagal stimulation of gastrin by GRP is via direct stimulation of the G cell and also partially by inhibition of the D cell (Debas and Carvajal, 1994).

Infusion of bombesin stimulated gastrin release in dogs (Bertaccini *et al.,* 1974; Modlin *et al.,* 1981), with larger molecular forms of GRP being more potent, possibly due to slower clearance (Bunnett *et al.,* 1985). Similar effects are observed in humans with bombesin (Varner *et al.,* 1980) or GRP infusion (Lundell *et al.,* 1987; Konturek *et al.,* 1992) and are unaffected or slightly enhanced by atropine (Fletcher *et al.,* 1983). In the rat stomach, bombesin stimulation of gastrin is insensitive to nicotinic and muscarinic blockade (Martindale *et al.,* 1982) and stimulates gastrin even when there is low gastric acidity (Matsuno *et al.,* 1997). Peptone meal stimulation of gastrin secretion is

partly mediated though GRP (Schubert *et al.,* 1992; Higham *et al.,* 1997). Thus, GRP is a neurotransmitter in both local and vagal gastrin stimulating mechanisms. Bombesin stimulates gastrin release from human antral G cells by activation of GRP receptor subtype-1 ($GRPR_1$) receptors (Squires *et al.,* 1999).

GRP/bombesin is also reported to stimulate somatostatin secretion. In the perfused rat stomach there are varying reports on how this occurs. Stimulation of somatostatin by GRP is reported to be inhibited by both hexamethonium and atropine (Martindale *et al.,* 1982), partially inhibited by atropine (Schubert *et al.* 1991), or atropine insensitive (Duval *et al.,* 1981; Azuma *et al.,* 1987; Gou *et al.,* 1987). Variation between results could be due to a number of factors. Firstly, the source of somatostatin is unclear in the perfused rat stomach. In rats, antral and fundic somatostatin are differentially regulated, but GRP stimulates fundic as well as antral somatostatin release (Schubert *et al.,* 1991). Secondly, cholinergic effects can stimulate acid secretion, which decreases gastrin secretion in response to bombesin (Modlin *et al.,* 1980). In the rat antrum, GRP stimulated somatostatin response via gastrin release (Schubert *et al.,* 1991). In contrast, bombesin had no effect on somatostatin secretion at 10^{-10}-10^{-6}M concentrations, but stimulated gastrin release in rat antral gland preparation (Richelson *et al.,* 1983). GRP receptors are present on D cells as well as G cells in a rat endocrine cell preparation (Zeng *et al.,* 1996), but the D cells could be fundic.

In pigs, bombesin directly stimulated somatostatin release, not via cholinergic effects or gastrin release (Holst *et al.,* 1987b, 1993). Bombesin over a range of 10^{-10}-10^{-6}M caused a dose-dependent stimulation of gastrin, but had no effect on somatostatin secretion from human antropyloric glands (Richelson *et al.,* 1983), or antral G and D cells in culture (Campos *et al.,* 1989, 1990), nor did bombesin stimulate antral D cells in culture (Buchan *et al.,* 1990). Bombesin receptors are not present on canine antral D cells (Vigna *et al.,* 1990). Thus it seems that while GRP releases somatostatin in pigs, this is not the case in humans, dogs nor, probably, in rats (Campos *et al.,* 1990).

In calves, infusion of GRP caused no significant change in serum gastrin concentration (Bloom *et al.,* 1983). In sheep, GRP, bombesin or ranatensin

infusion had no effect on gastrin secretion (Shulkes and Hardy, 1980; McDonald *et al.,* 1988; Shulkes *et al.,* 1994; Zavros *et al.,* 1999) or a variable effect (McLeay *et al.,* 1989) unless animals were actively immunised against somatostatin (Shulkes *et al.,* 1994; Zavros *et al.,* 1999). Somatostatin antiserum also increased the gastrin response to bombesin in the perfused rat stomach (Duval *et al.,* 1981). Thus, somatostatin may be responsible for variable GRP results in other species also (Shulkes *et al.,* 1994). GRP stimulated the integrated fundic somatostatin response, but decreased the antral response in sheep *in vivo*, with the fundic response said to dominate and inhibit gastrin secretion (Zavros *et al.,* 1999). Sheep antral tissue fragments showed a response to high doses of porcine GRP and bombesin, though bombesin was less effective (Lawton, 1995). Thus, while somatostatin responses to infused GRP/bombesin may differ slightly in sheep from monogastrics, the major neuronal stimulator of gastrin secretion is still GRP, as in other species.

1.1.7.2.2 Acetylcholine

Responses to cholinergic agonists and antagonists revealed both stimulatory and inhibitory effects *in vivo*. However, the majority of *in vitro* experiments suggest cholinergic effects on the G cell are stimulatory.

Pirenzipine inhibited cholinergic stimulation of gastrin release in the perfused rat stomach (Sue *et al.,* 1985) and rat antral tissue fragments (Harty *et al.,* 1988) suggesting gastrin release is via an M_1 receptor. Later studies suggest that M3 receptors mediate carbachol stimulated gastrin release from canine antral G cells (Yokotani *et al.,* 1995) and acetylcholine stimulated gastrin release in the rat stomach (Matsuno *et al.,* 1997). These conflicting results could be due to pirenzipine acting on M3 receptors (Yokotani *et al.,* 1995).

Cholinergic stimulation of gastrin release may be partially mediated through GABA. GABA is particularly concentrated in the antral mucosa and submucosa in rats, in neurons, in D cells (Harty *et al.,* 1991) and G cells (Davanger *et al.,* 1994). Harty *et al.* (1983, 1986, 1991) report that GABA stimulated gastrin and inhibited somatostatin release in rat antral mucosal fragments apparently through postganglionic cholinergic mechanisms. Weigert *et al.* (1998) reported inhibition of somatostatin secretion by GABA was via

cholinergic mechanisms, though mechanisms by which GABA stimulated gastrin secretion were unclear.

There are cholinergic pathways which stimulate gastrin release which are activated by the vagus. Stimulatory cholinergic pathways were revealed in dogs *in vivo* by the inhibition of stimulated gastrin secretion by high doses of atropine (Debas *et al.,* 1976; Hirschowitz *et al.,* 1979, 1981; Dockray and Tracy, 1980b; Eysselein *et al.,* 1985) and the stimulation of gastrin by bethanechol infusion (Hirschowitz and Gibson, 1978), but are masked by the inhibition of acid secretion with low doses, less than 25µg/kg, of atropine (Konturek *et al.,* 1974; Impicciatore *et al.,* 1977; Dockray and Tracy, 1980a, b; Katschinski *et al.,* 1992). In the perfused rat stomach, vagal stimulation at 2Hz released gastrin via a cholinergic pathway (Weigert *et al.,* 1993). There are also vagal pathways which inhibit gastrin secretion (Debas and Carvajal, 1994), though whether these act through cholinergic mechanisms is unclear.

Cholinergic pathways which stimulate gastrin release relate to luminal, not cephalic, pathways (Dockray and Tracy, 1980a, b). Gastrin responses to lumen conditions in rats are mediated, at least in part, by cholinergic neurons. Peptone meal stimulation of gastrin secretion is partly mediated by cholinergic neurons (Saffouri *et al.,* 1984b; Schubert *et al.,* 1992). Bile salts also stimulate gastrin via a cholinergic mechanism activated by capsaicin sensitive neurons (Miyata *et al.,* 1995).

In some species, cholinergic neurons stimulate gastrin release both directly and via inhibition of somatostatin release. In rat tissue *in vitro,* cholinergic agonists stimulate gastrin secretion (Harty and McGuigan, 1980) and also inhibit somatostatin secretion from perfused stomachs (Saffouri *et al.,* 1980; Duval *et al.,* 1981; Schubert *et al.,* 1982, 1985; Schubert and Makhlouf, 1982), antral tissue (Richelson *et al.,* 1983; Wolfe *et al.,* 1983; Sue *et al.,* 1985; Schubert *et al.,* 1987) and endocrine cell culture (Zeng *et al.,* 1996). Increased somatostatin levels inhibit carbachol-stimulated gastrin release (DeSchryver-Kecskmeti *et al.,* 1981; Wolfe *et al.,* 1983), suggesting cholinergic stimulation of gastrin is at least partly via inhibition of somatostatin. In sheep, bethanechol infusion decreased antral and fundic somatostatin and increased antral gastrin levels *in vivo* (Zavros *et al.,* 1999). This was not the case with sheep antral

tissue fragments however, in which carbachol stimulated gastrin release, but had no effect on somatostatin secretion, while bethanechol infusion had no effect (Lawton, 1995). In human G cell culture, muscarinic agonists did not stimulate gastrin secretion, but stimulated somatostatin secretion (Koop *et al.,* 1997). In contrast, carbachol stimulated gastrin release from human antropyloric glands (Richelson *et al.,* 1983).

A less widely accepted view, postulated in a review by Magee (1996), is that fundic-pyloric and pyloric-fundic reflexes exist, which are cholinergic. These cholinergic pathways were suggested by Magee (1996) to account for anomalies observed in various *in vivo* tests concerning the effects of atropine on the stomach. These reflexes are postulated to communicate between the antral G cell and the fundic parietal cell, dictating parietal cell sensitivity to gastrin which in turn controls gastrin secretion.

1.1.7.2.3 Catecholamines

There is evidence that gastrin secretion is partly mediated by adrenergic effects, with β-adrenergic agonists stimulating, and α-adrenergic agonists inhibiting, gastrin release. Early reports suggested that catecholamines stimulated gastrin release in humans (Hayes *et al.,* 1972; Brandsborg *et al.,* 1975, 1978) and dogs (Hayes *et al.,* 1978), but these studies did not account for acid secretion, which is inhibited via somatostatin by catecholamines (Koop *et al.,* 1980). Later studies show stimulatory β-adrenergic and possibly inhibitory α-adrenergic effects on G cells in humans (Peters *et al.,* 1982) and rats (Hayes *et al.,* 1978, DeSchryver-Kecskemeti *et al.,* 1981; Short *et al.,* 1985b; Harty *et al.,* 1988;).

In human antral G cells, β_2-adrenergic agonists are responsible for stimulating gastrin release and have synergistic effects with bombesin (Buchan, 1991). The β_3-adrenergic receptors have been characterised on rat D cells and G cells, but this does not rule out the presence of β_2-receptors (Levasseur *et al.,* 1997). In dogs, α_2-adrenoreceptor agonists decreased gastrin secretion also (Nakamura *et al.,* 1997).

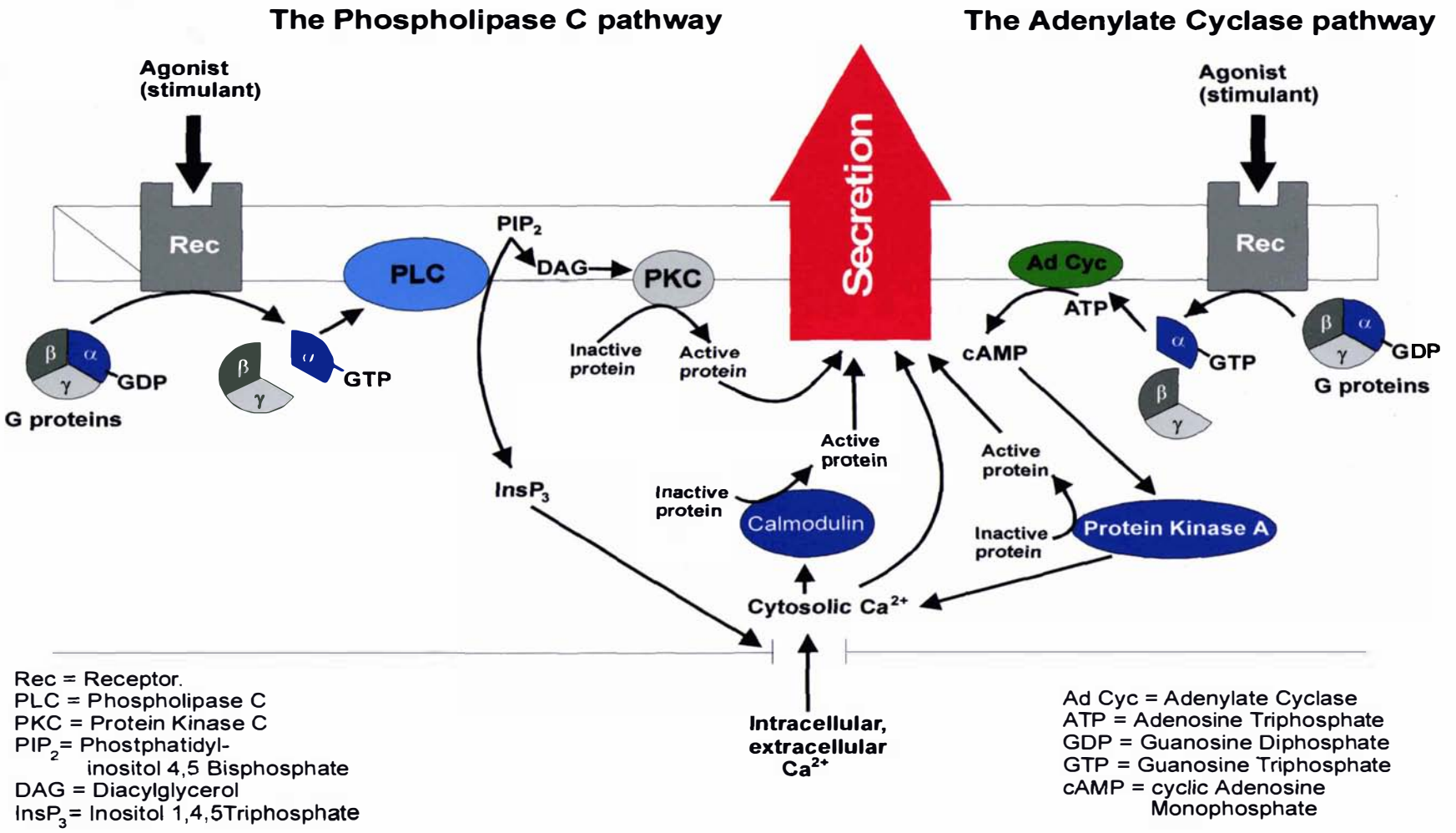

Figure 1.4. The second messenger pathways of the G cell controlling gastrin secretion. The processes of the Adenylate Cyclase and the Phospholipase C pathways are shown. These are affected by receptor action and interact and determine the overall secretory state of the G cell from all receptor action.

1.1.7.3 Signalling pathways

All signals received from membrane receptors, both stimulants and inhibitors, are integrated in the second messenger pathways in the G cell (Figure 1.4). Two second messenger pathways control the secretion of gastrin: the adenylate cyclase pathway and the phospholipase C pathway. Evidence for the activity of these pathways has been extensively studied. The activity of the adenylate cyclase pathways in controlling gastrin release is shown by the stimulation of gastrin secretion with cAMP (Gabrys-Pomykala *et al.,* 1977; Schebalin *et al.,* 1977; DeSchryver-Kesckemeti *et al.,* 1977 and 1981; Giraud *et al.,* 1987; Sandvik *et al.,* 1987; Sugano *et al.,* 1987a; Campos *et al.,* 1990; Shiotani and Merchant, 1994), but not cGMP (Schebalin *et al.,* 1977). Activation of Protein Kinase C and increased intracellular calcium cause gastrin release in canine (Giraud, 1987) and human (Campos *et al.,* 1990) antral G cells.

Bombesin-stimulated gastrin release is via Protein Kinase C in human antral G cells (Buchan *et al.,* 1994) and by calcium influx in human antral G cells (Buchan *et al.,* 1994), canine antral G cells (Sugano *et al.,* 1987a; Seensalu *et al.,* 1997) and the perfused rat stomach (Gou *et al.,* 1988), but not via cAMP (Sugano *et al.,* 1987a). Both activities were necessary for maximal effect in human antral G cells (Buchan *et al.,* 1994), but only calcium channels were activated in canine G cells (Seensalu *et al.,* 1997). The adenylate cyclase pathway also activates calcium channels. β-adrenergic stimulated gastrin release is mediated by L-type voltage dependent calcium channels in human antral G cells (Ray *et al.,* 1997b), proposed to be activated by cAMP dependent Protein Kinase A (Schubert, 1998). Peptide release by intracellular calcium in neurons is divided into a fast and a slow component (Kasai *et al.,* 1996; Gil *et al.,* 1998; Rupnik *et al.,* 2000). This may be true for G cells also.

As well as stimulating gastrin secretion, both cAMP (Shiotani and Merchant, 1995) and Protein Kinase C (Merchant *et al.,* 1991) stimulate gastrin mRNA production, at least in pituitary cells.

1.2 Gastrin secretion during abomasal parasitism

The pathology of nematode infection of the abomasum includes marked alteration in the morphology and secretions of the abomasal mucosa, especially in previously uninfected animals. The mucosal pathology in response to *O. circumcincta* infection in sheep was described in detail by Armour *et al.* (1966), with the principle findings confirmed in other studies of *O. circumcincta* (Sommerville, 1954; McLeay *et al.,* 1973; Anderson *et al.,* 1976a; Durham and Elliot, 1976; Elliot and Durham, 1976; Coop et al, 1977; Scott *et al.,* 1998b), *Haemonchus contortus* (Hunter and MacKenzie, 1982) infection in sheep, and *O. ostertagi* infection in cattle (Jennings *et al.,* 1966; Murray *et al.,* 1970). Typically, the normal secretory cells of the fundic gland are replaced by a hyperplastic population of cells of either minimal differentiation or a mucous cell phenotype. The pathology of abomasal nematode infection is commonly associated with the fundus (Armour *et al.,* 1966; Durham and Elliot, 1976; Elliot and Durham, 1976). However, Armour *et al.* (1966) reported lesions in the pylorus late in infection, Sommerville (1954) observed the infection was predominantly in the pylorus, while Durham and Elliot (1975) reported lesions could be primarily in either site.

Disruption of normal abomasal function by abomasal parasitism is apparent in three parameters: acid secretion, serum gastrin and serum pepsinogen. Other features of abomasal dysfunction include loss of sodium ions (McLeay *et al.,* 1973) and albumin into the lumen along with serum hypoalbuminaemia, hypocalcaemia and hypophosphataemia (reviewed by Holmes, 1993; McKellar, 1993). Gut motility is also decreased (reviewed by Fox, 1997). Hypergastrinaemia is thought to play a central role in the host response, causing anorexia (Fox *et al.,* 1989a; Fox, 1997), decreased gut motility (Fox, 1997), increased pepsinogen secretion (Fox *et al.,* 1989a) and later aiding the recovery of acid secretion (Scott *et al.,* 1998a).

Hypergastrinaemia occurs in sheep infected with *O. circumcincta* (Anderson *et al.,* 1976b, 1981, 1985, 1988; Blanchard and Westcott, 1985; Lawton *et al.,* 1996; Scott *et al.,* 1998a, 2000) or *H. contortus* (Blanchard and Westcott, 1985; Nicholls *et al.,* 1988; Simpson *et al.,* 1997) and *O. ostertagi* infection in calves (Fox *et al.,* 1987, 1989a, b, 1993; Snider *et al.,* 1988;

Purewal *et al.,* 1997). Antrectomy abolished the gastrin response to infection, suggesting the antrum is the source of increased gastrin secretion (Anderson *et al.,* 1981).

Hypergastrinaemia has been proposed as a diagnostic tool for Ostertagiasis in cattle, along with pepsinogen (Hilderson *et al.,* 1992; Berghen *et al.,* 1993). While serum pepsinogen has routinely been used to diagnose abomasal parasitism, some sheep secrete very little pepsinogen and show poor pepsinogen responses to infection (Lawton *et al.,* 1996; Scott *et al.,* 1998c). Therefore, as serum pepsinogen alone may not be reliable for the diagnosis of abomasal parasitism, hypergastrinaemia could be used as an additional indicator. Hypergastrinaemia could be an accurate indicator of parasitism in cattle, in which a strong correlation between serum gastrin and abomasal pH has been demonstrated (Fox *et al.,* 1993).

Pathological conditions in the stomach inducing hypergastrinaemia have been extensively studied in humans, as have the effects of omeprazole-induced hypoacidity on gastrin secretion in rats. Most conditions which induce gastric pathology in humans, including pernicious anaemia, duodenal ulceration and *H. pylori* infection, induce hypergastrinaemia. There may be similarities between hypergastrinaemia in these diseases and the hypergastrinaemia observed during abomasal parasitism in ruminants. Omeprazole-induced hypergastrinaemia in rats is also a useful model for examination of gastrin under hypoacidity, as is induced by abomasal parasitism in ruminants.

1.2.1 Effects of hypergastrinaemia

1.2.1.1 Anorexia

Anorexia is probably the most serious detrimental effect of abomasal parasitism (Symons, 1985; Coop and Kyriazakis, 1999) and may be related to hypergastrinaemia (reviewed by Fox, 1997). Decreased food intake has been frequently reported in sheep infected with *O. circumcincta* (Parkins *et al.,* 1973; Anderson *et al.,* 1976a; Sykes and Coop, 1977) or *H. contortus* (Abbot *et al.,* 1986a, b) and in cattle infected with *O. ostertagi* (Fox *et al.,* 1989a). Both cholecystokinin (Symons, 1985) and gastrin (Fox *et al.,*1989a; Fox, 1997) have

been proposed as causative agents in the development of anorexia during gastrointestinal parasitism. Hypergastrinaemia has been associated with reduced weight gain in *O. ostertagi* infected calves (Fox *et al.,* 1989b, Hilderson *et al.,* 1992). This is supported by experiments using omeprazole treatment in calves, which resulted in similar serum gastrin concentrations to those observed in calves during *O. ostertagi* infections, and were accompanied by a 40% reduction in food intake (Fox *et al.,* 1989a, Fox, 1997). Similarly, chickens treated with omeprazole exhibited anorexia, which reversed after treatment with a gastrin antagonist (Campbell *et al.,* 1991), showing hypergastrinaemia is responsible for reduced food intake during omeprazole treatment. How hypergastrinaemia affects appetite is not clear. CCK_A receptors, not CCK_B receptors, in the central nervous system are responsible for cholecystokinin mediated inhibition of food intake (reviewed by Fink *et al.,* 1998; Ritter *et al.,* 1999). Central blockade of CCK_A has been shown to increase food intake in *Trichostrongylus colubriformis* infected lambs (Dynes *et al.,* 1998), suggesting this receptor is involved in anorexia induced by parasitism. It is not clear whether gastrin could affect food intake by binding to central CCK_A receptors as, typically, gastrin has poor affinity for CCK_A receptors. Another mechanism by which gastrin may inhibit food intake in ruminants is via inhibition of reticuloruminal motility, which could in turn result in stasis of digesta in the abomasum and rumen, causing reduced food intake (Fox *et al.,* 1989a).

1.2.1.2 Hyperpepsinogenaemia

Gastrin may also stimulate increased pepsinogen secretion during abomasal infection. Hyperpepsinogenaemia accompanies *O. circumcincta* infection in sheep (McLeay *et al.,* 1973; Coop *et al.,* 1977; Lawton *et al.,* 1996; Scott *et al.,* 1998b) and *O. ostertagi* infection in cattle (Anderson *et al.,* 1966; Jennings *et al.,* 1966; Murray et al, 1970; Fox *et al.,* 1987). Gastrin has been shown to stimulate gastrin release in monogastrics (Hersey *et al.,* 1983; Kleveland *et al.,* 1987; Cherner *et al.,* 1988; Lin *et al.,* 1992; Lanas *et al.,* 1994; Blandizzi *et al.,* 1999), and hyperpepsinogenaemia correlates with hypergastrinaemia after omeprazole treatment in cattle (Fox *et al.,* 1989a). Hypergastrinaemia may contribute to hyperpepsinogenaemia during periods of markedly elevated abomasal pH in the presence of adult parasites (Armour *et*

al., 1966; Jennings *et al.,* 1966; Anderson *et al.,* 1985; McKellar *et al.,* 1986, 1987a; Lawton *et al.,* 1996). However, there are small increases in serum pepsinogen prior to (Lawton *et al.,* 1996) or even without (McKellar *et al.,* 1987a) increased abomasal pH. The increase in pepsinogen secretion in response to hypergastrinaemia induced by omeprazole treatment is less than that observed during parasitism (Fox *et al.,* 1989a). These results suggest that hypergastrinaemia is not solely responsible for hyperpepsinogenaemia (Fox *et al.,* 1989a). Other proposed causes of hyperpepsinogenaemia are: increased leakage through more permeable mucosa and decreased conversion to pepsin (Jennings *et al.,* 1966; Murray *et al.,* 1970); stimulation of release by parasite secretions (McKellar *et al.,* 1990b); a hypersensitive response in previously infected animals (Berghen *et al.,* 1993; Scott and McKellar, 1998).

1.2.1.3 Hyperplasia

The hypergastrinaemia during abomasal parasitism has been suggested to have positive trophic effects on the abomasal mucosa, aiding parietal cell recovery (Scott *et al.,* 1998a). Hypergastrinaemia has also been associated with the fundic hyperplasia and increase in mucosal weight during *O. circumcincta* in sheep (Anderson *et al.,* 1988). In parasite free calves, omeprazole-induced hypergastrinaemia results in hyperplasia, supporting the hypothesis that gastrin may by partly responsible for fundic hyperplasia during abomasal nematode infection (Fox, 1997). However, changes in the mucosal cell populations during parasitism are more like those observed with overexpression of EGF peptides than the typical trophic actions of gastrin (reviewed by Simpson, 2000).

1.2.2 Gastrin biosynthesis, tissue gastrin and blood gastrin during hypersecretion

Prolonged hypergastrinaemia typically results in a proportionate increase in processing intermediates in the antral tissue, along with an upregulation of transcription and post-translational processing. Although hypergastrinaemia has been related to changes in abomasal pH during the course of a parasite infection, the effects of continued hypergastrinaemia on G cell function during

parasitism have not been extensively studied. However, the responses of the G cell to continued stimulation induced by gastric hypoacidity in rats are well studied, and are probably similar in response to all stimuli. The overall response of the G cell to continued stimulation by achlorhydria is graded: firstly, there is an increase in gastrin secretion; then translation of gastrin mRNA and post translational processing increases; followed by an increase gastrin mRNA transcription; finally, G cell numbers increase.

Hypergastrinaemia during abomasal helminth infections may have similarities to the hypergastrinaemia induced by *H. pylori* infection in humans, which has been intensively studied. In contrast to helminth infections in ruminants, *H. pylori* infection may increase acid secretion depending upon the site of infection (McColl *et al.,* 1997). However, the cause of hypergastrinaemia induced by *H. pylori* infection may be similar to that occurring with abomasal helminth infections of ruminants. *H. pylori* infection increases antral juxtamucosal pH (Kelly *et al.,* 1993), which may induce hypergastrinaemia. In addition, the hypergastrinaemia produced by antral *H. pylori* infection is also associated with gastric damage and inflammation (Sankey *et al.,* 1990). Both hypoacidity and inflammation may contribute to the hypergastrinaemia with abomasal helminth infection. Thus, effects observed on G cells with *H. pylori* infection also relate to hypergastrinaemic cells during *Ostertagia* infection.

1.2.2.1 G and D cell numbers

In general, prolonged hypergastrinaemia is associated with an increase in G cell numbers, a decrease in D cell numbers, or both, leading to a reduction in the G:D cell ratio. A 14 day omeprazole treatment resulted in increased G cell numbers (Dockray *et al.,* 1991), while 10 week (Allen *et al.,* 1986) or 20 week (Larrson *et al.,* 1988) treatment with omeprazole increased G cell numbers, G:D cell ratio and decreased D cell numbers. In duodenal ulcer patents, increased G cell numbers (Polak *et al.,* 1978) and increased G:D cell ratios have been reported (Polak *et al.,* 1978; Kishimoto *et al.,* 1985; Sumii *et al.,* 1994; Annibale *et al.,* 1996), as have typical G cell numbers (Creutzfeldt *et al.,* 1976; Arnold *et al.,* 1982; Graham *et al.,* 1993), typical G:D cell ratios (Arnold *et al.,* 1982; Graham *et al.,* 1993), and even decreased G:D cell ratios (Chamouard *et al.,* 1997). These varied results may partly be due to the

degree of gastritis, as severe gastritis results in a reduction in G cell numbers, possibly due to tissue damage (Sankey *et al.,* 1990) or reduced visibility of G cells due to hypersecretion (Marotta *et al.,* 1990).

In both sheep infected with *O. circumcincta* (Scott *et al.,* 1998a) and calves infected with *O. ostertagi* (Purewal *et al.,* 1997), hypergastrinaemia is accompanied by a reduction in G cell numbers, not G cell hyperplasia. The absence of hyperplasia could be a result of the period of hyperplasia being too short. The shortest reported period omeprazole treatment in rats which induced G cell hyperplasia appears to be 14 days by Dockray *et al.* (1991). Although increased G cell numbers have been reported within two days of truncal vagotomy, this was due to increased gastrin production and staining, not hyperplasia (Shimoda *et al.,* 1990). On the other hand reduced G cell numbers may be due to weak staining of G cells due to hypersecretion (Marotta *et al.,* 1990; Fox *et al.,* 1993).

1.2.2.2 Transcription and post translational processing

The timing of increased transcription and post translational processing of gastrin has been extensively studied in rats, but has not been studied in detail in ruminants with parasite-induced hypergastrinaemia. It seems likely that increased transcription and rate of post-translational processing responses of the G cell to prolonged hypergastrinaemia are very similar regardless of the stimulus, whether induced by hypoacidity due to omeprazole treatment or parasitism.

Gastrin production increases to accommodate increased secretion. Initially, there is an increase in gastrin secretion, followed within 24 hours by an increase in gastrin mRNA translation (Bate *et al.,* 1996), then after about two days an increased mRNA transcription (Macro *et al.*, 1997). Reversal of increased synthesis also lags behind reversal of hypergastrinaemia (Dockray *et al.,* 1993). The timing of gastrin mRNA increases in response to omeprazole treatment appear to be similar in sheep, where increases in gastrin mRNA lag behind increased gastrin secretion by at least 24 hours (Read *et al.,* 1993). Gastrin mRNA levels were increased in response to *O. ostertagi* infection in cattle after several days of hypergastrinaemia (Purewal *et al.,* 1997). Thus,

increased gastrin production in response to hypergastrinaemia induced by parasitism appears to be similar to that reported with hypoacidity in rats.

In rats, the rate of post translational processing of gastrin is also increased in response to increased gastrin secretion. Phosphorylation and amidation are key reactions in the processing of the gastrin peptides, controlling progastrin and amidated gastrin formation (see 1.1.4.2). Both phosphorylation and amidation rates are reduced with decreased gastrin synthesis in fasted rats (Varro *et al.,* 1990b, c; Dimaline *et al.,* 1991) and with decreased secretion rats with antral exclusion in dogs (Varro *et al.,* 1990a). However, pulse-chase experiments showed that pre-treatment of rats with omeprazole did not increase the rate of gastrin amidation nor cleavage at the Arg57-Arg58 or the Arg94-Arg95 sites (Macro *et al.,* 1997). Thus, amidation and cleavage or progastrin appear not to be upregulated during hypersecretion.

Pulse-chase experiments in rat antral G cells showed the timing of post translational events using radiolabelled peptides (Varro *et al.,* 1995). Progastrin was first observed after 30 minutes, with G34Gly, then G34 appearing at 60 minutes with G17 forms becoming apparent after 120 minutes (Varro *et al.,* 1995). Turnover of cellular content is estimated to occur once every four hours, suggesting about half of G17's residency time in the cell is during its formation (Dockray *et al.,* 1996).

As conversion of G34 to G17 is the slowest of the processing steps, its upregulation is critical in hypersecretory states, where residency time of gastrin peptides in G cells is reduced. Omeprazole treatment in rats results in an increase in the cleavage of G34 to G17 (Macro *et al.,* 1997), probably via upregulation of PC2. There is also an increase in PC1/3 and PC2 mRNA after five day omeprazole treatment (Macro *et al.*, 1996), showing that gastrin processing enzymes are also upregulated during hypersecretion. A 24 hour treatment with omeprazole in rats caused an increased rate of conversion of G34 to G17 and G34Gly to G17Gly, possibly regulating hypergastrinaemia by reducing amounts of G34 (Macro *et al.,* 1997). This upregulation of dibasic cleavage may also be due to increased processing rates to match levels of secretion.

Processing may also be affected by external factors, such as gastric luminal constituents. Cleavage of G34 to G17 is dependent on the acidity of

the secretory vesicle (Voronina *et al.,* 1997), which may be affected by luminal factors. Dietary amines as well as biogenic amines, raise the vesicle pH and inhibit G34 cleavage to G17 (Hussain *et al.,* 1999). Whether this can become a significant factor during gastrin hypersecretion is unknown.

Despite faster processing of gastrin peptide, hypersecretion results in lower residency time of peptides in the G cell, resulting in less complete peptide processing before secretion and thus, processing intermediates becoming more abundant in blood and account for greater proportions of the gastrin peptides in the G cell. Hypersecretion of gastrin in sheep after 24 hours omeprazole infusion resulted in an increased proportion of G34 compared to G17 in blood, a slight decrease in G17 in the antrum, and similar proportions of G-Gly and amidated gastrins in both blood and antral tissue (Read *et al.,* 1993). In humans, continued gastrin hypersecretion was also characterised by increased amounts of G34, component I and G-Gly in serum, accompanied in the tissue by an increased concentration of G-Gly, but no change in the ratio of G17 and G34 (Jensen *et al.,* 1989). Similar results were observed in both sheep (Scott *et al.,* 1998a) and calves (Fox *et al.,* 1993; Purewal *et al.,* 1997) parasitised with *Ostertagia*, in which increased serum gastrin comprised mostly of G34, while G17 predominated in control animals, but the ratio of G17 to G34 was unchanged in antral tissue. In calves, the increased serum gastrin concentration was shown to be due to increased gastrin synthesis (Purewal *et al.,* 1997). This is also true in omeprazole treated rats, in which increased secretion contributes more to increased serum gastrin levels than increased tissue gastrin content (Dockray *et al.,* 1991).

In both sheep infected with *O. circumcincta* (Scott *et al.,* 1998a) and calves infected with *O. ostertagi* (Purewal *et al.,* 1997) and subject to hypergastrinaemia, tissue gastrin concentration was reduced. These results were not consistent with results in omeprazole-treated, hypergastrinaemic rats, in which tissue gastrin, particularly G-Gly, were increased (Dockray *et al.,* 1991). This may relate to the period of elevation of pH, in that tissue gastrin levels were estimated after some seven to eight days of hypergastrinaemia in the studies of both Scott *et al.* (1998a) and Purewal *et al.* (1997), while those of Dockray *et al.* (1991) were conducted after 14 days of hypoacidity and

presumably hypergastrinaemia. Thus, increased gastrin synthesis and processing may take some time to compensate for an initial reduction in tissue gastrin as a result of hypersecretion.

Both Scott *et al.* (1998a) and Purewal *et al.* (1997) suggest the increased proportions of G34 in blood could be due to the longer half-life of G34, and its preferential release from G cells. Scott *et al.* (1998a) suggest that increased proportions of G34 in the gastroepiploic vein of uninfected sheep support its preferential release. While preferential secretion of immature vesicles in response to stimulation does occur with some peptides, generally preferential secretion does not occur in response to secretion stimulants (Arvan and Castle, 1998). Preferential secretion of immature vesicles has not been observed in most studies of gastrin hypersecretion. There is a greater proportion of immature vesicles, containing both G34 and G17, in the G cell during hypersecretory states (Alumets *et al.,* 1979, 1980; Hakanson *et al.,* 1982), and after feeding (Track *et al.,* 1978) suggesting shorter residence time for vesicles in G cells, with no evidence of storage vesicles or preferential secretion of immature vesicles. While tissue proportions of G17 and G34 do not change during gastrin hypersecretion (Jensen *et al.,* 1989; Purewal *et al.,* 1997; Scott *et al.,* 1998a) this could be due to increased conversion of G34 to G17 during hypersecretion (Macro *et al.,* 1997). The increased proportion of G34 in the gastroepiploic vein could be due to postsecretory metabolism of G17 (see 1.1.5.1). If the endopeptidases responsible for postsecretory cleavage obey Michaelis-Menten kinetics, and basal gastrin levels are below the Michaelis constant for these enzymes, it is possible that their activity increases as gastrin secretion increases accounting for the higher proportions of G34 in circulation.

1.2.3 Causes of hypergastrinaemia

1.2.3.1 Abomasal hypoacidity

Hypoacidity during abomasal nematode infection is likely to be the primary cause of hypergastrinaemia. In calves there is very good correlation between hypoacidity and hypergastrinaemia (Fox *et al.,* 1993). In sheep, the

relationship between hypoacidity and hypergastrinaemia appeared weaker, but both increase together, initially at least (Lawton *et al.,* 1996).

Lawton *et al.* (1996) reported that gastrin secretion increased with abomasal pH within 24 hours of transplant of adult *O. circumcincta* into sheep. Although gastrin responses to cephalic stimulation occur within minutes in rats (Track *et al.,* 1979) and dogs (Dockray *et al.,* 1980b), induction of increased gastrin release by gastric hypoacidity takes up to one hour to develop (Kline *et al.,* 1975; Shulkes and Hardy, 1982a).

Hypoacidity is commonly observed with abomasal parasitism by *O. circumcincta* (Armour *et al.,* 1966; McLeay *et al.,* 1973; Anderson *et al.,* 1976a, b, 1981, 1985, 1988; Titchen and Anderson, 1977; Lawton *et al.,* 1996; Scott *et al.,* 1998a, b), *H. contortus* (Christie *et al.,* 1970; Nicholls *et al.,* 1987, 1988; Simpson *et al.,* 1997) and cattle with *O. ostertagi* (Jennings *et al.,* 1966; Fox *et al.,* 1993). Elevated abomasal pH may be necessary for nematode survival, as worms suspended in the normal abomasum (McKellar, 1993; Simpson *et al.,* 1999) or in acidic medium *in vitro* (Haag, 1995), die rapidly. On the other hand, the strong buffering capacity of the gastric mucus (Schreiber and Scheid, 1997) may be sufficient to protect worms from gastric luminal acidity.

In calves, hypergastrinaemia and abomasal hypoacidity are observed with *O. ostertagi* infection after emergence of adult worms, with hypergastrinaemia strongly correlated to increased abomasal pH (Fox *et al.,* 1993). Furthermore, omeprazole infusion in unparasitised calves produces hypergastrinaemia similar to abomasal parasitism (Fox *et al.,* 1989a). This is in keeping with the commonly accepted model, that gastric acidity controls antral gastrin secretion, described in Section 1.1.6.2 2. In sheep, however, Anderson *et al.* (1976b, 1981, 1985) claimed plasma gastrin was elevated before abomasal pH increased in sheep infected with *O. circumcincta.* This interpretation was dependent on the threshold of hypoacidity being pH4. This threshold was considered too conservative by Lawton *et al.* (1996). Instead Lawton *et al.* (1996) defined hypoacidity as any value two standard deviations above the mean pH for pre-infection values. Using this definition, Lawton *et al.* (1996) observed that serum gastrin and abomasal pH increased in unison, but, when abomasal pH increased beyond pH5.5, serum gastrin levels returned to

near basal levels, only to increase again when abomasal pH declined. This latter feature was also noted in some animals by Anderson *et al.* (1985). It seems fluctuations in abomasal pH alone cannot explain gastrin secretion in sheep during *O. circumcincta* infection.

Abomasal hypoacidity coincides with the loss of differentiated epithelial cells in the fundus, which is symptomatic of abomasal Ostertagiosis (Sommerville, 1954; Armour *et al.,* 1966). Parietal cell loss occurs in two patterns depending upon the maturity of the *Ostertagia* worm. The L3 larvae cause local epithelial cell hyperplasia and parietal cell loss early in the infection when they invade the mucosa (Sommerville, 1957; Armour *et al.*, 1966), but, generally, cause little change in abomasal pH. Adult worms cause more global epithelial damage, resulting in the most severe increase in abomasal pH and parietal cell loss after completion of the third ecdysis (Armour *et al.,* 1966; Denham, 1969) and emerging from the mucosa (Sommerville, 1954, 1963). The pattern of parietal cell loss is similar in cattle infected with *O. ostertagi*, although the emergence of adults occurs much later, between days 17-21 (Murray *et al.,* 1970). Abomasal pH begins to decrease again with the expulsion of adult worms, which occurs at a similar time to when gravid females and patency are reported (Denham, 1969; McLeay *et al.,* 1973; Lawton *et al.,* 1996).

It was previously suggested that larval invasion of and emergence from the mucosa are the chief causes of pathophysiology and abomasal hypoacidity (Armour *et al.,* 1966; Jennings *et al.,* 1966; Murray *et al.,* 1970). More recent studies, however, suggest that the presence, not the emergence, of adult worms causes the most significant changes in abomasal secretion (Lawton *et al.,* 1996; Simpson *et al.,* 1997; Scott *et al.,* 1998a, b). Transplant of adult *O. circumcincta* has consistently been demonstrated to elevate abomasal pH and serum gastrin, as quickly as within 24 hours, to levels equivalent to those observed with adult emergence with larval infections (Anderson *et al.,* 1985; Lawton *et al.,* 1996; Scott *et al.,* 2000). Adult *Ostertagia* are not associated with tissue invasion, suggesting that a chemical interplay is central to inhibition of acid secretion.

Reduced abomasal acidity, which may result in hypergastrinaemia, could be due to a reduction in parietal cell activity and reduced parietal cell numbers. Parietal cells with ultrastructure indicating reduced activity were observed in cattle infected with *O. ostertagi* (Murray *et al.,* 1970) and sheep infected with *O. circumcincta* (McLeay *et al.,* 1973; Scott *et al.,* 2000). Reduced parietal cells numbers are also reported after larval *O. ostertagi* infection in cattle (Murray *et al.,* 1970) and after both larval and adult *O. circumcincta* infection in sheep (Scott *et al.,* 1998b, 2000).

The actual cause of reduced parietal cell activity is unknown. Hypergastrinaemia often coincides with hypoacidity, which suggests that stimulation of gastrin secretion is being blocked, either at the level of the parietal cell or the ECL cell (Hertzberg *et al.,* 2000). There are two candidates for this effect, parasite secretions or inflammatory effects. The role of inflammation is supported by the coincidence of decreased parietal cell numbers and abomasal hypoacidity with the accumulation of eosinophils and neutrophils during *O. circumcincta* infection in sheep (Scott *et al.,* 1998b, 2000). Scott *et al.* (1998b) suggest that the host growth factors, such as TGFα, or chemicals released by adult parasites may be the cause of acid inhibition. While there is no published evidence connecting growth factor levels during parasitism and acid inhibition, worm secretions and homogenates have been studied. McKellar *et al.* (1990a) reported *O. ostertagi* secretions had no effect on acid secretion from dispersed bovine fundic cells, but *O. ostertagi* homogenate inhibited acid secretion when injected in rats (McKellar, 1993). However, ammonia secretion from *O. circumcincta in vitro* inhibited acid secretion in dispersed rabbit fundic glands (Scott, Merkelbach and Simpson, unpublished results). Interestingly, in rat stomachs, topical administration of ammonia increases EGF production and secretion (Brzozowski *et al.,* 1996; Konturek, 1997), which in turn inhibits parietal cell function and stimulate the growth of epithelial surface cells (Dockray, 1999).

1.2.3.2 Inflammation, inflammatory mediators and gastrin secretion

Although hypoacidity appears to be the major stimulant of hypergastrinaemia induced by abomasal nematode parasitism in sheep, inflammation of the mucosa may also be a contributing factor. There is a

greater increase in serum gastrin levels during abomasal parasitism than is observed during infusion of bicarbonate, or rumen contents into the abomasum increasing pH (Reynolds *et al.,* 1991). This suggests that another factor, in addition to elevated abomasal pH, is involved in parasite-induced hypergastrinaemia. Intestinal parasitism has been reported to induce gastrin hypergastrinaemia in rats (Castro *et al.,* 1976), possibly via inflammation. An inflammatory response, involving migration of eosinophils and neutrophils to the mucosa, was observed during *O. circumcincta* infection when the larvae first infected the mucosa and progressed throughout infection (Armour *et al.,* 1966). Scott *et al.* (1998b, 2000) observed that migration of eosinophils to tissue coincided with abomasal hypoacidity. Globular leukocytes are also noted in the mucosa later in infection (Armour *et al.,* 1966), and are associated with rejection of worms. While Hertzberg *et al.* (1995) reported immunosuppression in sheep previously exposed to parasites had no effect on gastrin levels, the possible acute effects of inflammatory cytokines on gastrin secretion in parasitised sheep have not been studied in detail.

There are no direct studies on the effects of inflammatory mediators on gastrin secretion in sheep, or during abomasal parasitism, though the role of such mediators has been studied in relation to *H. pylori* infection. *H. pylori* infection increases the release of inflammatory cytokines, which may stimulate prostaglandin release, activate inflammatory cells and stimulate gastrin release (Lehmann and Salder, 1998). Several cytokines have been shown to stimulate G cells in various species. Rabbit antral G cells are stimulated by Interleukin 1β (IL1β) and TNFα (Weigert *et al.,* 1996). Canine antral G cells are stimulated by monocyte and mononuclear cell secretions, and by TNFα, Interferon-γ, and IL2, but not IL1β, and not at the concentrations secreted by monocyte/mononuclear cell incubation (Lehmann *et al.,* 1996). In contrast, Beales *et al.* (1997) reported canine antral G cells were stimulated by IL8, but not by TNFα. *H. pylori* sonicates potentiated gastrin release by IL8, which stimulates gastrin secretion via a receptor, an effect which could be inhibited by somatostatin (Beales *et al.,* 1997). Increased amounts of IL6 and TNFα are secreted in culture by the antral mucosa of *H. pylori* infected gastritis patients (Crabtree *et al.,* 1991). With *H. pylori* positive and negative human antral

fragments, TNFα increased gastrin stimulation by bombesin and decreased gastrin inhibition by cholecystokinin (Beales and Calam, 1997).

G cells may be stimulated or inhibited by prostaglandins. Gastrin secretion was inhibited by prostaglandin E_2 (PGE_2) in perfused rat stomach (Saffouri *et al.,* 1980) and by enprostil in humans (Mahachai *et al.,* 1985; Tari *et al.,* 1997). Inhibition may be via somatostatin release in the perfused rat stomach (Saffouri *et al.,* 1980). This is not the case with canine antral cell culture, where enprostil inhibited and PGE_2 both stimulated and inhibited gastrin secretion, independent of somatostatin (Schepp *et al.,* 1994). Prostaglandins also inhibit the release of inflammatory mediators from mast cells (Hogaboam *et al.,* 1993) as well as acid secretion (Konturek *et al.,* 1980; Mahachai *et al.,* 1985), both of which may affect gastrin secretion. The reported effects of prostaglandin analogues may be pharmacological rather than physiological however, as gastric damage induced by indomethacin did not affect gastrin or acid secretion in humans (Rademaker *et al.,* 1995).

1.2.3.3 G cell sensitivity

Increased G cell sensitivity may contribute to hypergastrinaemia during abomasal parasite infection in similar fashion to *H. pylori* infection in humans, which appears to alter the physiological control of gastrin. In *H. pylori* infected duodenal ulcer patients, the G cell is more sensitive to stimulation by bombesin/GRP (Graham *et al.,* 1991; Beardshall *et al.,* 1992; Gibbons *et al.,* 1997) and gastric alkalinsation (El Nujumi *et al.,* 1998; Jensen *et al.,* 1987), but less sensitive to acidification (Jensen *et al.,* 1987). *H. pylori* infection increases basal gastrin levels (Ohkusa *et al.,* 1997) with decreased somatostatin levels (Gibbons *et al.,* 1997). The increased sensitivity of the G cell may be due to reduction of the somatostatin inhibitory feedback effect. *H. pylori* infected duodenal ulcer patients also exhibit increased gastrin and somatostatin mRNA production in response to GRP, probably due to abnormalities in the somatostatin feedback on gastrin (Gibbons *et al.,* 1997). Inhibition of gastrin and acid secretion by cholecystokinin, which acts via somatostatin, is defective in duodenal ulcer patients, as blockade of cholecystokinin receptors had no effect on gastrin or even acid secretion (Konturek *et al.,* 1995). Jensen *et al.* (1987) also questioned the inhibitory effect of somatostatin on gastrin release in

duodenal ulcer patients, as somatostatin levels in the right gastroepiploic vein were unaffected by altered gastric pH. A similar effect may occur in *O. circumcincta* as hypergastrinaemia coincides with a reduction in the number of D cells (Scott *et al.,* 1998a).

1.2.3.4 Nematode excretory/secretory products and gastrin secretion

It has also been suggested that excretory/secretory products (E/S) of *Ostertagia* stimulate gastrin secretion (McLeay *et al.,* 1973; McKellar *et al.,* 1987a), as in some infection studies, hypergastrinaemia occurs without hypoacidity. However, Lawton (1995) and Haag (1995) both reported E/S products from *Ostertagia* cultured *in vitro* had no effects on gastrin secretion from ovine antral fragments.

It is possible that the both the hypoacidic and hypergastrinaemic response to a nematode infection involves ammonia. *O. circumcincta* releases ammonia at concentrations sufficient to inhibit parietal cells (Scott, Merkelbach, Simpson, unpublished results). Ammonia, produced by urease activity, is thought to contribute to *H. pylori* induced hypergastrinaemia (Levi *et al.,* 1989). Ammonia production allows *H. pylori* to function in low pH media (Rektorshek *et al.,* 1998). Ammonia may be responsible for the observed increase in antral juxtamucosal pH, which may impair the inhibitory feedback of acid on gastrin secretion (Kelly *et al.,* 1993). Neither urea infusion (Chittajallu *et al.,* 1991a; Graham *et al.,* 1991), nor urease inhibition (El Nujumi *et al.,* 1991) nor reduced urease activity (Chittajallu *et al.,* 1991b) affected gastrin levels in *H. pylori* positive duodenal ulcer patients though. In contrast, long-term dietary ammonium loading in rats increased mucosal thickness, tissue and serum gastrin levels, gastrin mRNA levels and serum gastrin response to a meal (Litchtenberger *et al.,* 1995; Dial *et al.,* 1996) and gastrin sensitivity to ammonia in food (Dial *et al.,* 1996). Some of these effects at least may be due to gastric injury and inflammation (Lichtenberger *et al.,* 1995). Mononuclear phagocytes and cytokine production are also stimulated by *H. pylori* urease (Harris *et al.,* 1996).

Various products isolated in parasite E/S products have been isolated which may affect gastrin secretion. Hertzberg *et al.* (1999) observed an elevation in serum gastrin after anthelmintic treatment in trickle infected sheep,

possibly produced by dying worms releasing antigens. In cattle, the size of the adult worm is thought to have an effect on the level of gastrin secretion (Berghen *et al.,* 1993). This may relate to either a secretory capacity of the worm or the antigenic response of the host. Acetylcholinesterase activity has been isolated in many studies of nematode parasites, including in the infective larvae of *O. circumcincta* (Sutherland and Lee, 1993), and from the E/S of *T. colubriformis* (Griffiths and Prichard, 1994). *Ostertagia* worms have also been shown to release a bombesin like peptide (Huntington *et al.,* 1999). Both of these may affect gastrin secretion directly. Other effects of parasite E/S products are reported also. For example, *O. osteragi* secrete an eosinophil chemotactic factor (Klesius *et al.,* 1986), *O. ostertagi* homogenate (McKellar, 1993) and *O. circumcincta* E/S (Scott, Merkelbach and Simpson, unpublished results) inhibit acid secretion, while *Teladorsagia circumcincta* E/S stimulates HT29-D4 cell proliferation (Huby *et al.,* 1995). Such effects could affect gastrin secretion indirectly.

1.2.3.5 Abomasal microbes

A novel suggestion, put forward by Lawton *et al.* (1996) is that abomasal bacteria present in the hypoacidic abomasum during parasite infection may affect gastrin secretion. Both Lawton (1995) and Haag (1995) reported that when incubation cultures of *O. circumcincta* were contaminated with abomasal bacteria, an inhibitory effect on *in vitro* gastrin secretion was observed. This factor may also be present in the hypoacidic abomasum of parasitised sheep, which contains increased bacterial numbers. Jennings *et al.* (1966) reported increased aerobic bacterial numbers in the abomasa of calves infected with *O. ostertagi*, and proposed that their survival may be the cause of the diarrhoea. It is expected however, that aerobic bacteria are only a small percentage of the bacterial population in the abomasum, as they are in the rumen. Nicholls *et al.* (1987) showed greatly increased numbers of anaerobic bacteria in the abomasum during *H. contortus* infection in sheep. Increased anaerobe survival coincided with increased abomasal pH and decreased pO_2 in the abomasum.

These studies provide some support for the statement by Lawton *et al.* (1996), that an overgrowth of bacteria at high abomasal pH could interfere with gastrin secretion. It is has been extensively reported that *H. pylori* infection in

humans affects gastrin secretion, such infection stimulates gastrin secretion. Lawton *et al.* (1996) proposed that microbes surviving in the abomasum during *O. circumcincta* infection were inhibiting gastrin secretion, based *in vitro* observations. These observations show a novel effect of bacteria on gastrin secretion from the antrum.

1.3 Summary and aims

The control of gastrin release in the monogastric is well defined, even in disease states. In contrast, there are relatively few studies on the control of gastrin secretion in ruminants. In particular, there appears to be only one reported study of gastrin secretion by ruminant abomasal tissue *in vitro,* that of Lawton (1995). Further, experiments relating to gastrin secretion in ruminants may have been affected by abomasal parasitism. In this thesis, experiments are reported which examine the control of gastrin secretion from unparasitised antral tissue, with particular emphasis on the inhibition of gastrin secretion by somatostatin and the stimulation of gastrin release by luminal chemicals (Chapter 2).

In ruminants, the study of gastrin secretion is centred around hypergastrinaemia during abomasal parasitism. Although abomasal pathophysiology during abomasal parasite infection is well described, the factors affecting gastrin release and the effects of gastrin hypersecretion are still debated. The experiments reported in Chapter 3 and 4 were designed to investigate further the role of abomasal hypoacidity as the primary cause of increased serum gastrin concentrations and to examine the effects of hypergastrinaemia in *O. circumcincta*-infected sheep, in particular on food intake. The presence of abomasal microbes and their effects on gastrin secretion were examined (Chapter 3), as was the presence and effects of an inhibitor of *in vitro* gastrin secretion in abomasal contents (Chapter 4). The origin, basic properties and probable nature of the microbial product which inhibits *in vitro* gastrin secretion were also examined (Chapters 5 and 6).

CHAPTER 2

PHARMACOLOGY OF THE UNPARASITISED OVINE ANTRAL G CELL

2.1 Introduction

Regulation of gastrin secretion by the ruminant antrum appears to involve mechanisms similar to those reported in monogastric species, which have been extensively studied. The complex regulation of this hormone by neural, chemical and paracrine factors has been reviewed in Chapter 1.

Cephalic stimulation of gastrin release is mediated by the vagus (Dockray and Tracy, 1980a, b; Hirschowitz and Fong, 1990), mainly via a direct effect of GRP on the G cell (Schubert *et al.,* 1985; Weigert *et al.,* 1993; Debas and Carvajal, 1994), but also through reduced somatostatin secretion via inhibitory cholinergic neurons (Debas and Carvajal, 1994). Chemical stimulation of gastrin release by gastric peptones is also mediated through GRP and cholinergic neurons (Saffouri *et al.,* 1984b; Schubert *et al.,* 1992). Conversely, amino acids and dietary amines act directly on the G cell to stimulate gastrin release (Lichtenberger *et al.,* 1982a, b; Dial *et al.,* 1986, 1991; DelValle and Yamada, 1990).

Somatostatin, a well established inhibitor of gastrin release, plays a key role in regulating the activity of the G cell. The inhibitory effect of gastric acid feedback is mediated by increased somatostatin release (Schudziarra *et al.,* 1978; Schubert *et al.,* 1988; Greenberg *et al.,* 1993) in response to stimulation by nerves containing CGRP (Ren *et al.,* 1992; Manela *et al.,* 1995). In rats, the effects of distension on gastrin secretion vary according to magnitude: gastrin secretion is reduced by VIP-stimulated somatostatin release during mild distension, whereas cholinergic neurons stimulate gastrin release during moderate distension (Schubert and Makhlouf, 1993). In humans, distension causes gastrin release by a β-adrenergic mechanism (Peters *et al.,* 1982). Both positive and negative somatostatin responses are amplified by a negative feedback loop between somatostatin and histamine in the antrum of rats

(Vuyyuru *et al.,* 1995). Other chemicals and inflammatory mediators are postulated to have effects on gastrin release, though their precise role is uncertain.

In vivo studies on ruminants have suggested that GRP nerves may be less important in stimulating release of gastrin than in monogastrics and that somatostatin restraint of basal gastrin release was particularly high. Infusion of GRP or bombesin was ineffective in stimulating gastrin release in calves (Bloom *et al.,* 1983) and sheep (Shulkes and Hardy, 1980; McDonald *et al.,* 1988), unless sheep were immunised against somatostatin (Shulkes *et al.,* 1994; Zavros *et al.,* 1999). Infusion of somatostatin into sheep increased (Barry *et al.,* 1985; Reynolds *et al.,* 1991) or decreased (Shulkes and Hardy, 1982a) basal plasma gastrin levels, whereas omeprazole-induced hypergastrinaemia was not associated with a change in plasma somatostatin (Read *et al.,* 1992). The discrepancies between studies may have partly arisen due to concurrent effects on acid secretion.

Lawton (1995) developed an *in vitro* method to study gastrin secretion by ovine tissue. Results obtained were, in general terms, similar to those reported for monogastric tissues: GRP, but not bombesin was the most potent stimulant of gastrin release, whereas carbachol weakly stimulated gastrin release. Somatostatin secretion was elevated by VIP and GIP, but this elevated somatostatin secretion did not affect basal gastrin release. This latter result was attributed to basal somatostatin levels exerting maximal inhibition on basal gastrin secretion. However, it is unclear from these experiments whether somatostatin played any role in controlling gastrin secretion.

One factor not controlled in the *in vitro* studies of Lawton (1995) was whether the antral tissue was taken from an abomasum subject to parasitism. Gastritis due to ammonia (Dial *et al.,* 1996) or *H. pylori* infection (Graham *et al.,* 1991; Beardshall *et al.,* 1992; Annibale *et al.,* 1996; Gibbons *et al.,* 1997) in monogastric animals enhances the sensitivity of the G cell to stimulation. Abomasal parasitism may cause a similar increase in G cell sensitivity, as parasitised abomasal tissue often exhibits inflammation (Armour *et al.,* 1966; McLeay *et al.,* 1973; Stear *et al.,* 1996; Scott *et al.,* 2000).

Experiments described in this chapter examined the sensitivity of G cells

from unparasitised tissue to stimulants, the role of somatostatin in controlling gastrin release and the sensitivity of ovine G cells to chemical stimulation by amino acids.

2.2 Methods

2.2.1 *In vitro* incubation method

Gastrin and/or somatostatin responses to pharmacological agents were determined from the ratio of peptide released in the presence of the agent to that previously released in control medium. Each tissue piece thus acted as its own control and removed variation due to the size of the tissue or its overall gastrin or somatostatin content.

2.2.1.1 Incubation medium

The basal incubation medium was Hank's Balanced Salt Solution (HBSS) without $NaHCO_3$ (GIBCO BRL, Life Technoiogies Inc., USA), to which was added 0.35g.L^{-1} $NaHCO_3$, 0.25% Bovine Serum Albumin (Fraktion V, Boehringer Mannheim GmbH, Deutschland), 0.1% D-glucose (Sigma Chemical Co., USA) and 10mM HEPES (GIBCO BRL, Life Technologies Inc., USA), adjusted to pH 7.40±0.02 and 320-340mOsm.L^{-1}.

Gassing solutions with oxygen or carbogen (95% oxygen, 5% CO_2) prior to dispensing for incubation did not result in an increase in oxygen concentrations above atmospheric levels at the time of incubation. Gassing solutions with carbogen resulted in a reduction in basal gastrin secretion, probably due to a reduction in medium pH. Continuous gassing of solutions during incubation was impractical. Therefore solutions were not gassed prior to incubation, as gassing with oxygen was ineffectual and gassing with carbogen was detrimental to basal gastrin secretion.

For each test (treatment plate), eight pieces of mucosa were incubated at 37°C, first for 30 minutes (3x10 minute incubations) in control medium (A solution) followed by 30 minutes (3x10 minute incubations) in the test solution (B solution). The calculated secretory response to the test solution was adjusted for the slow decline in secretion of control tissue by using the simultaneous

incubation of 24 or 32 reference tissue pieces in control medium for the whole 60 minutes (control plates).

2.2.1.2 Tissue

All sheep from which tissue was taken were removed from pasture, orally dosed with 0.2mg.kg^{-1} Ivermectin (Merial, New Zealand Ltd.) seven days prior to the experiment, then housed indoors and fed on a lucerne chaff diet. Sheep were euthanased with either 10mL Pentobarb 500 (Chemstock Animal Health, Christchurch, NZ) or stunned by captive bolt and exsanguinated. The abomasum was immediately removed, the mucosa was washed gently with control medium, separated from underlying tissue by blunt dissection and cut into segments 3-4mm square. Eight pieces were placed on pins and mounted on a polystyrene strip, spaced so that each piece could be suspended in the centre of a well along one side of a 48 multi-well plate (Costar Corporation, USA).

2.2.1.3 Assay procedure

After a 30 minute equilibration period in control medium in a 37°C room, the tissues were transferred to the test plates and moved at 10 minute intervals through the six rows of wells; the first three contained 1mL of control medium and the second three contained control fluid with or without the test agent. At the completion of the incubation, the plates were kept for two to four hours at 4°C until the three incubates for each solution were aspirated and pooled for gastrin assay; a 1mL subsample was placed into tubes containing 10µl of Trasylol (Bayer, Germany) for somatostatin assay. All samples and subsamples were stored at -20°C until assayed. The treatment response was then calculated by dividing the ratio of [B solution] / [A solution] for the treatment plate by the average [B solution] / [A solution] ratio for the control plates, then expressing the overall response as a percentage change versus control secretion.

2.2.2 Pharmacological agents

The following pharmacological agents were tested at the concentrations stated (n= the number of experiments).

Porcine GRP-28 (pGRP, G1649, Sigma Co., USA) 10^{-6}M and 10^{-7}M (n=6).

Human GRP (hGRP, G8022, Sigma Co. St., USA) 10^{-6}M and 10^{-7}M (n=4).

Vasoactive Intestinal Peptide (VIP, V3628 Sigma Chemical Co. St., USA) at 10^{-6}M (n=2).

Gastric Inhibitory Polypeptide (GIP, G5512 Sigma Chemical Co. St., USA) at 10^{-6}M and 10^{-7}M (n=6).

Nicotine (free base, N3876, Sigma Chemical Co., USA) from $5x10^{-4}$M to 10^{-6}M (n=5).

Somatostatin-14 (S9129, Sigma Chemical Co., USA) from 10^{-6}M to 10^{-9}M (n=3).

Histamine (H7250, Sigma Chemical Co., USA) from 10^{-3} to 10^{-9}M (n=3).

Carbachol (C4382, Sigma Chemical Co., USA) from 10^{-2} to 10^{-4}M (n=4).

Atropine (A0257, Sigma Chemical Co., USA) at 10^{-6}M (n=2).

Adrenaline (David Bull Laboratories, NZ) from 10^{-4}M to 10^{-8}M (n=3).

L-Glycine (G7126, Sigma Chemical Co., USA) from 10^{-2}M to 10^{-4}M (n=2).

L-Phenylalanine (P2126 Sigma Chemical Co., USA) from 10^{-2}M to 10^{-4}M (n=3).

L-Tryptophan (T0254 Sigma Chemical Co., USA) $5x10^{-3}$M to 10^{-4}M (n=3).

VIP at 10^{-6}M in combination with 10^{-6}M porcine GRP (n=2).

GIP at 10^{-6}M in combination with 10^{-6}M or 10^{-7}M GRP (n=6).

Nicotine at $5x10^{-4}$ to 10^{-6}M in combination with 10^{-6}M somatostatin (n=3).

2.2.3 Statistical analyses

All data are presented as the mean±standard-error of the mean (SEM) for a single experimental plate (eight pieces of tissue) unless otherwise indicated. Positive values indicate a stimulation of secretion over control levels, while negative values represent an inhibition of secretion, producing results below basal secretion; "n" is the number of experiments in which each drug or drug combination was tested.

Experiment	Basal levels (pM)	Agent	Effect
1	445± 94	GRP	No effect
	308±17	VIP	44% stimulation*
	317±119	GRP/VIP	21% stimulation*
2	428±72	GRP	No effect
	396±64	GRP/VIP	16% stimulation
3	459±47	$5x10^{-3}$N	53% stimulation
	406±91	$5x10^{-4}$M to $1x10^{-6}$M N	No effect
	396±138	hGRP	36% stimulation
4	518±41	$5x10^{-3}$N	28% inhibition
	362±56	$5x10^{-4}$N	No effect
	376±10	$1x10^{-4}$N	32% inhibition
	329±14	$1x10^{-5}$N	28% inhibition
	289±18	$1x10^{-6}$N	No effect
	289±25	hGRP	18% inhibition
5	4024±1235	GRP	No effect
	4474±1061	GIP	No effect
	4345±1180	GRP/GIP	No effect
6	2371±512	GRP	No effect
	3654±1028	GIP	No effect
	4881±1384	GRP/GIP	No effect
7	1108±307	10^{-3}M C	No effect
8	541±145	10^{-3}M to 10^{-8}M H	No effect
9	202±97	10^{-3}M H to 10^{-8}M H	No effect
	369±222	10^{-3}M C	No effect
10	438±53	10^{-1}M H	No effect
	672±59	10^{-2} to 10^{-6}M H	30-40% inhibition
	683±72	10^{-4}M C	40% inhibition
	552±113	10^{-2}M, 10^{-3}M, 10^{-6}M C	No effect
11	443±93	$5x10^{-4}$-10^{-6}M N	No effect
12	362±41	$5x10^{-4}$-10^{-6}M N	No effect

Table 2.1. Somatostatin secretion (mean±SD) for plates in 12 individual pharmacology experiments, prior to the action of the pharmacological agent. Pharmacological agents are GRP = GRP 10^{-6}M, GIP = GIP 10^{-6}M, VIP = VIP 10^{-6}M, N = Nicotine, C = Carbachol, H = Histamine. Only significant ($p<0.05$) effects are shown.

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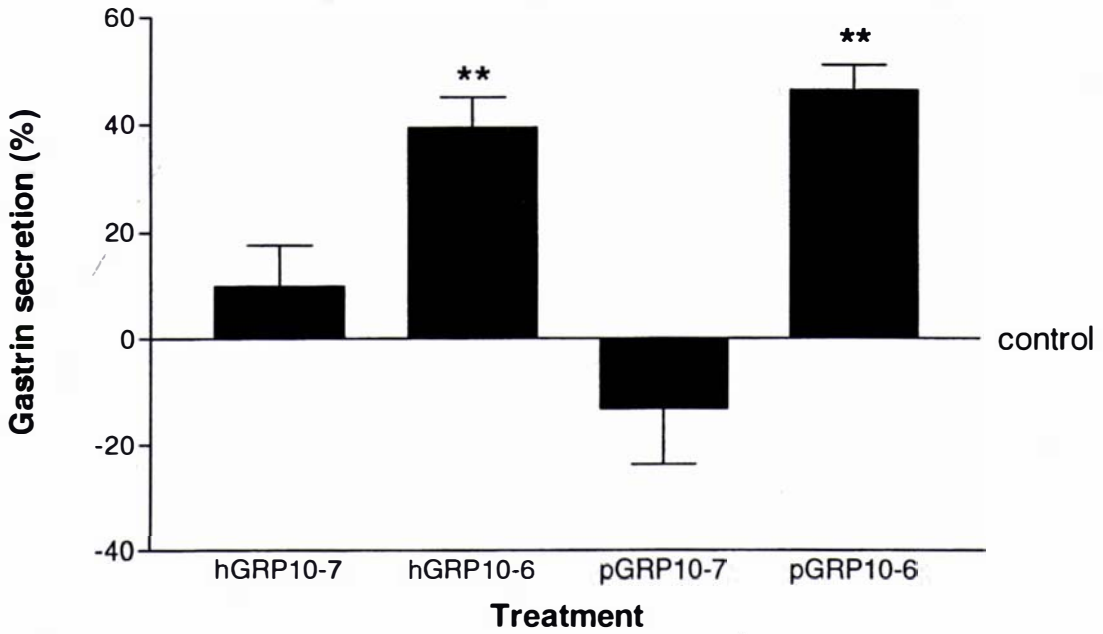

Figure 2.1. Effects of human (hGRP) and porcine (pGRP) GRP at 10^{-6}M and 10^{-7}M concentration on gastrin secretion. Data expressed as mean±SEM, ** indicates results are different from control, $p<0.01$.

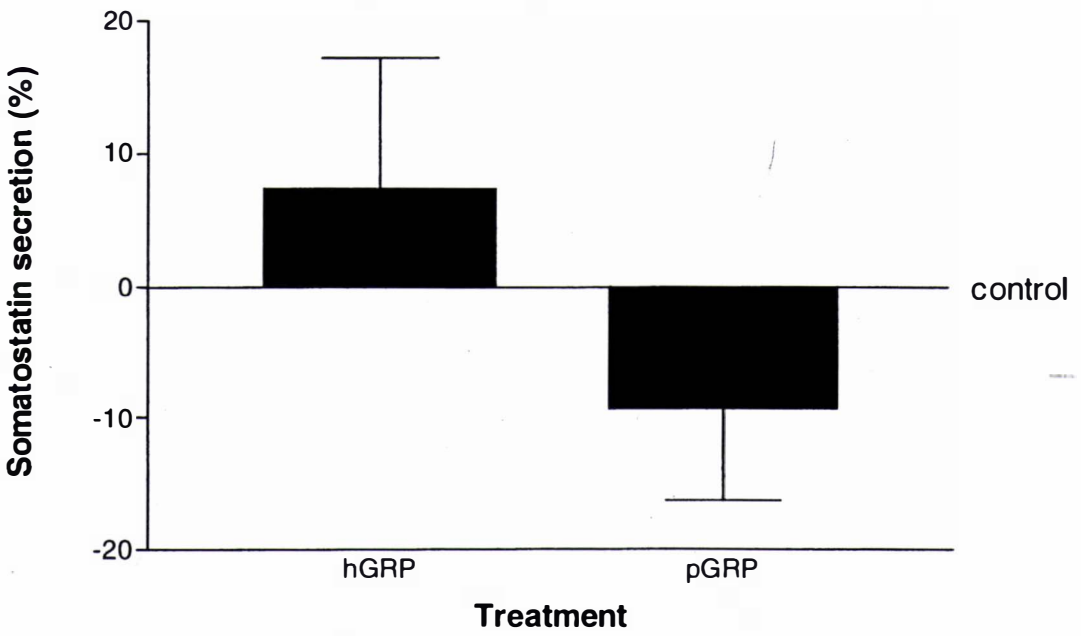

Figure 2.2. Effects of human (hGRP) and porcine (pGRP) at 10^{-6}M concentration on somatostatin secretion. Data expressed as mean±SEM.

All results were analysed using SPSS version 9.0, using Univariate Analysis of Variance (UNIANOVA), which considered treatment (for example, the dosage of drug used) and experimental date (experiments on different days used different tissues) as sources of variation. All data were examined for normality using the Shapiro-Wilks test when groups contained fewer than 50 values, and the Kolmogorov-Smirnov test when groups contained more than 50 values. Data sets in comparisons were further tested for equality of variance using Levene's Homogeneity of Variation test. *Post hoc* comparisons of data groups were conducted using Tukey's HSD test for comparisons of all groups or Dunnett's test when compared with control alone, and variances were equal. When variances were unequal, Tamhane's T2 *post hoc* test was used. In cases where only two groups were compared, data were similarly analysed for normality and equality of variance and independent samples t-tests were conducted. When the data were not normally distributed, Kruskal Wallis analysis in Graphpad Prism version 2.01, with Dunn's *post hoc* test were used.

2.3 Results

Gastrin concentrations in A solutions were between 100pM and 400pM. The decline in gastrin concentrations in B control, solutions compared to A solutions was between 10% and 40%, but was mostly around 25%. The average concentration of somatostatin in A solutions is shown in Table 2.1.

2.3.1 GRP

Both pGRP (n=6) and hGRP (n=4) stimulated gastrin secretion at 10^{-6}M ($p<0.01$), but had no significant effect ($p>0.05$) at 10^{-7}M (Figure 2.1). The responses to 10^{-6}M pGRP and hGRP ranged from 28±4% to 82±6% and 1±7% to 63±12% respectively. In general, the responses to porcine or human GRP forms were not different ($p>0.05$). Responses did vary between experiments ($p<0.0001$) for both types of GRP, suggesting that the tissues varied in the sensitivity to GRP.

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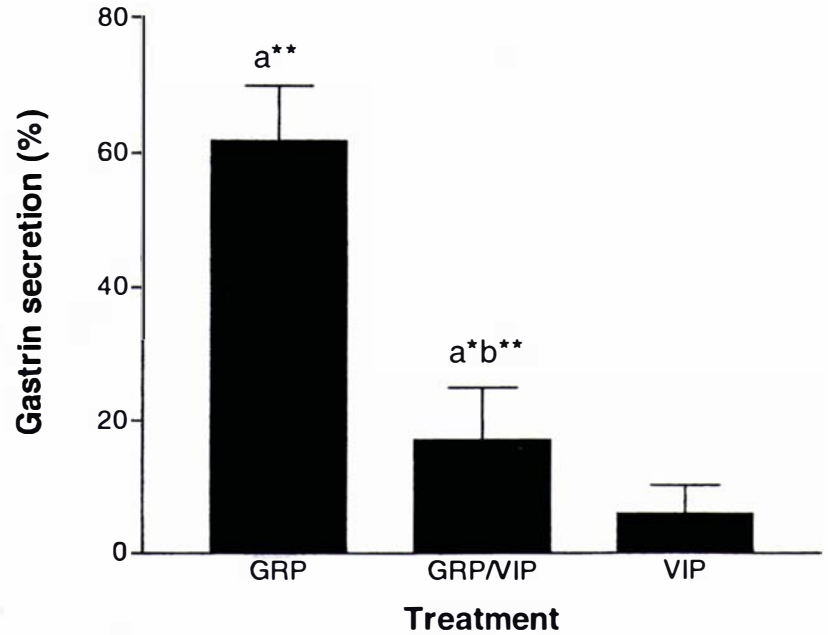

Figure 2.3. Effect of VIP at 10^{-6}M on basal and10^{-6}M porcine GRP-stimulated gastrin release. Data expressed as mean±SEM, a= secretion greater than control, b= secretion less than GRP-stimulated response, *=p<0.05, **=p<0.01.

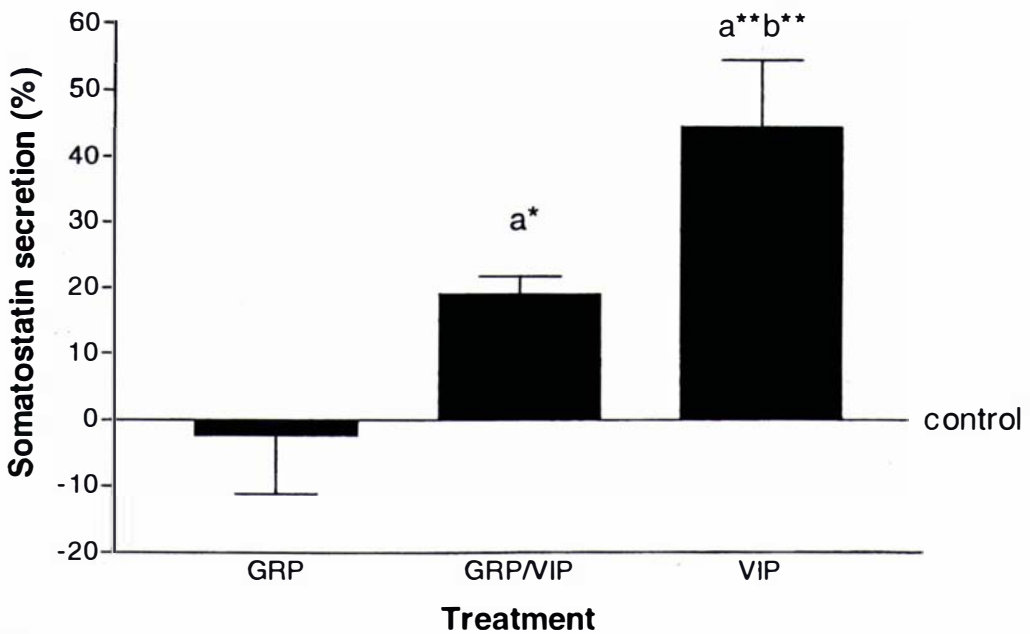

Figure 2.4. Effects of 10^{-6}M VIP on somatostatin secretion, alone and in the presence of 10^{-6}M porcine GRP. Data expressed as mean±SEM, a= secretion greater than control, b= secretion greater than GRP response *=p<0.05, **=p<0.01.

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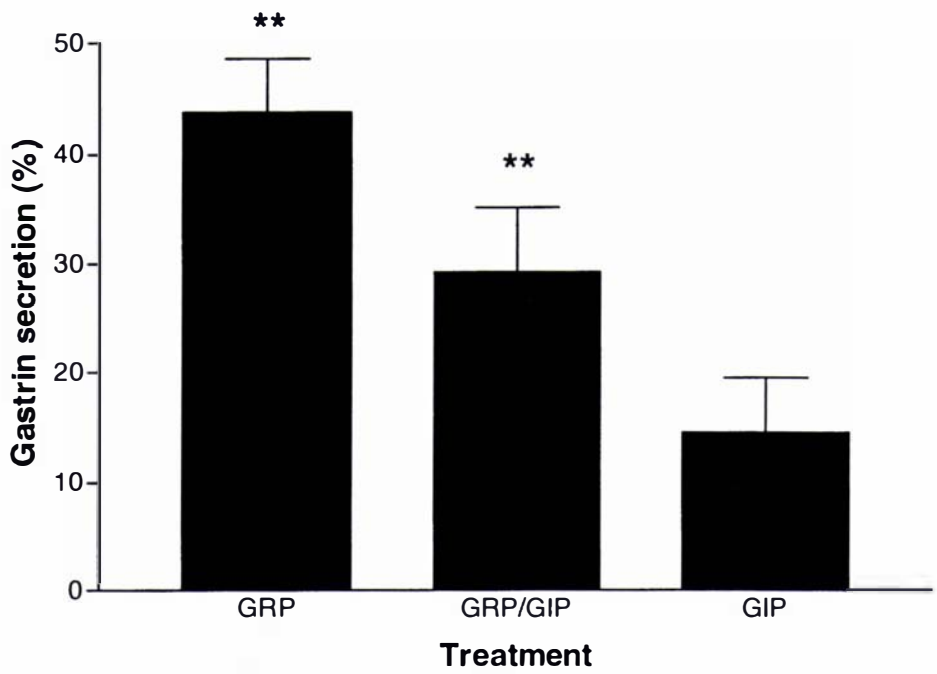

Figure 2.5. Effects of 10^{-6}M GIP on basal and 10^{-6}M porcine GRP-stimulated gastrin release. Data expressed as mean±SEM, **=p<0.01, gastrin secretion is greater than control.

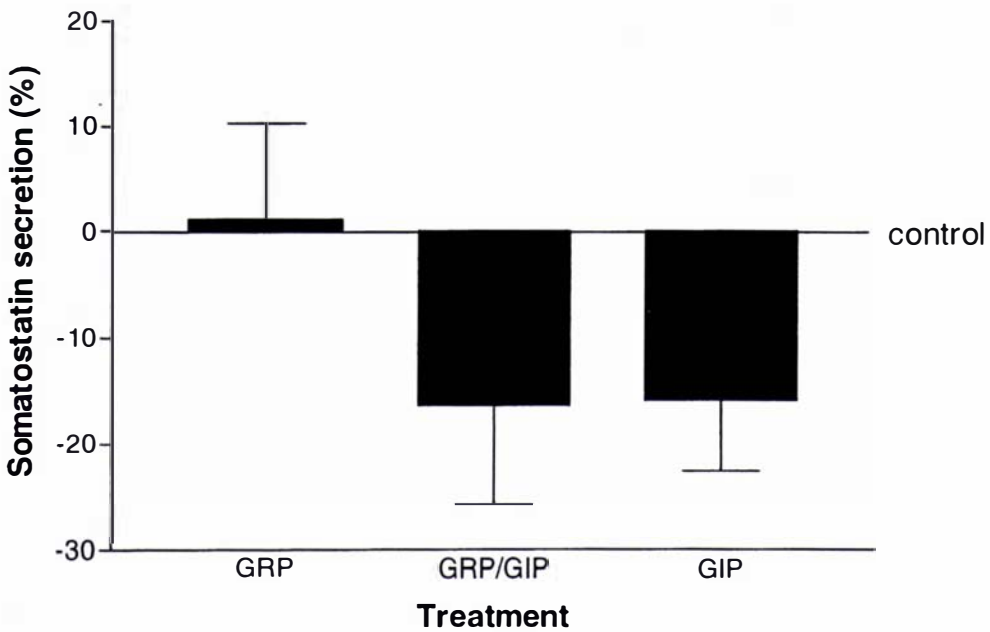

Figure 2.6. Effect of 10^{-6}M GIP on somatostatin secretion, alone and in the presence of 10^{-6}M porcine GRP. Data expressed as mean±SEM.

Neither pGRP (n=4) nor hGRP (n=2) at 10^{-6}M affected somatostatin secretion ($p>0.05$) when results from individual experiments were pooled (Figure 2.2). Somatostatin responses to 10^{-6}M pGRP in individual experiments ranged from 6±6% to -24±10%, none of which were significant ($p>0.05$). However, the two experiments which tested the somatostatin response to hGRP 10^{-6}M, an 18±3% inhibition ($p<0.05$) in one experiment and 36±14% stimulation ($p<0.05$) in the other. In the experiment in which hGRP 10^{-6}M inhibited somatostatin secretion, the gastrin response was greater ($p<0.05$) than in the experiment in which 10^{-6}M hGRP stimulated somatostatin release. There was significant variation ($p<0.001$) between experiments in somatostatin responses to porcine or human GRP.

2.3.2 VIP with GRP

VIP at 10^{-6}M (n=2) had no effect ($p>0.05$) on basal gastrin release (Figure 2.3), but stimulated ($p<0.01$) somatostatin release by 45±10% (Figure 2.4). In both experiments, 10^{-6}M VIP decreased 10^{-6}M pGRP-stimulated gastrin release ($p<0.01$). Somatostatin secretion was significantly elevated ($p<0.05$) by 20±2% above control levels in the presence of both 10^{-6}M VIP and 10^{-6}M pGRP. However, this response was not significantly different ($p>0.05$) from the somatostatin responses to 10^{-6}M GRP or 10^{-6}M VIP alone. Somatostatin responses to 10^{-6}M VIP were similar in both experiments ($p>0.05$).

2.3.3 GIP with GRP

The overall gastrin response to 10^{-6}M GIP (n=6) of 29±10% was not significant ($p>0.05$, Figure 2.5), nor was somatostatin secretion affected by 10^{-6}M GIP ($p>0.05$, Figure 2.6).

GIP at 10^{-6}M (n=6) did not affect ($p>0.05$) 10^{-6}M pGRP-stimulated gastrin secretion, and did not alter ($p>0.05$) somatostatin levels when combined with 10^{-6}M pGRP (Figure 2.6). There was significant variation ($p<0.001$) in gastrin secretion between experiments, due to the variable gastrin response to pGRP

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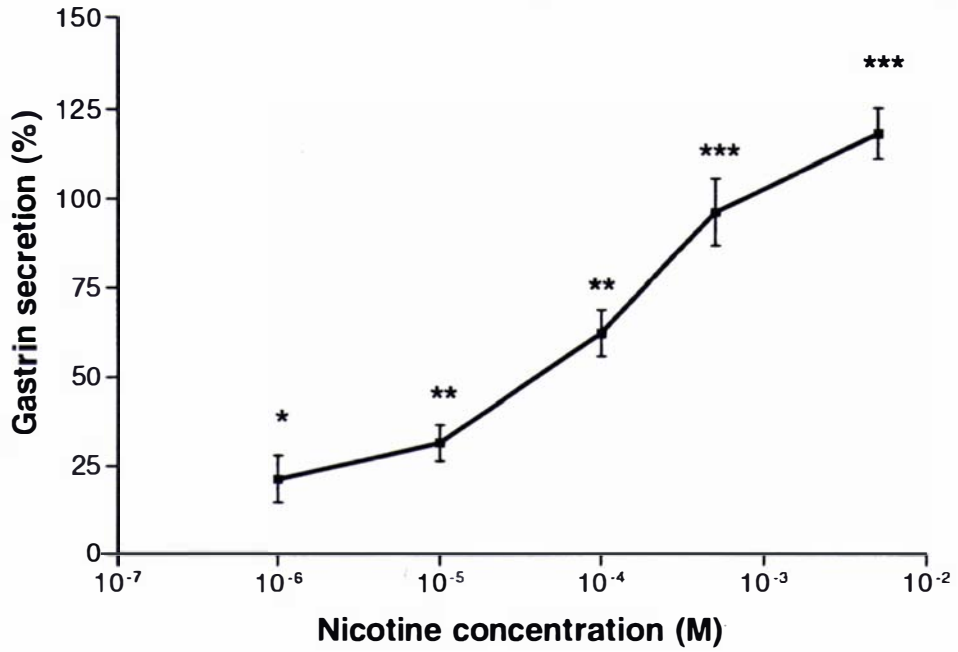

Figure 2.7. Gastrin response to nicotine at concentrations from $5x10^{-3}$ to $1x10^{-6}$M. Data expressed as mean±SEM, *=p<0.05, **p<0.01, ***=p<0.001, gastrin secretion greater than control.

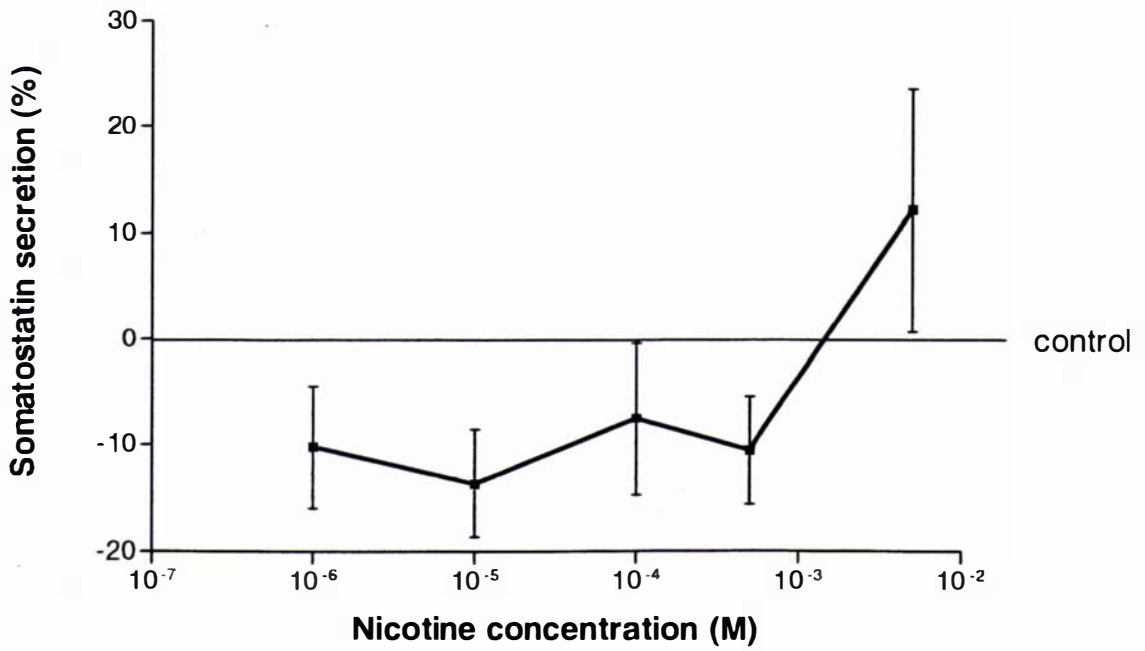

Figure 2.8. Somatostatin response to nicotine at concentrations from of $5x10^{-3}$ to $1x10^{-6}$M. Data expressed as mean±SEM.

(+28±4% to +82±6%) and GIP (-25±9% to +38±13%). There was no variation in the somatostatin response to GRP or GIP between experiments ($p>0.05$).

2.3.4 Nicotine

Nicotine (n=5) caused a dose-dependent increase in gastrin release (Figure 2.7), but had no significant effect ($p>0.05$) on somatostatin levels (Figure 2.8). On two occasions, hGRP and nicotine were used to stimulate gastrin release with tissue from the same animal. In both experiments, the stimulation of gastrin secretion in response to the highest nicotine concentrations ($5x10^{-3}M$ and $5x10^{-4}M$) was greater than the stimulation of gastrin secretion induced by hGRP at $10^{-6}M$ ($p<0.01$). There was no significant difference ($p>0.05$) in the gastrin response to nicotine in four of the five experiments conducted. In one experiment, however, the gastrin response to all concentrations of nicotine was lower ($p<0.05$) than in the other four experiments.

The somatostatin response to nicotine was variable ($p<0.001$) between the four experiments in which it was tested. Somatostatin secretion was not examined in the experiment during which consistently lower gastrin responses to nicotine were observed, when compared with the other four experiments in which gastrin response to nicotine was tested. In one experiment, nicotine inhibited ($p<0.05$) somatostatin secretion at concentrations of $5x10^{-3}M$, $10^{-4}M$ and $10^{-5}M$ concentrations. In another experiment, $5x10^{-3}M$ nicotine stimulated somatostatin release ($p<0.05$). These two experiments were the same experiments as those described in Section 2.3.1, in which variable somatostatin responses to hGRP $10^{-6}M$ were observed. As with the hGRP-stimulated responses, gastrin secretion was higher ($p<0.05$) in the experiment in which somatostatin secretion was inhibited by nicotine than in the experiment in which somatostatin was stimulated. No significant effects were observed ($p>0.05$) in the other two experiments in which the effect of nicotine on somatostatin secretion was tested.

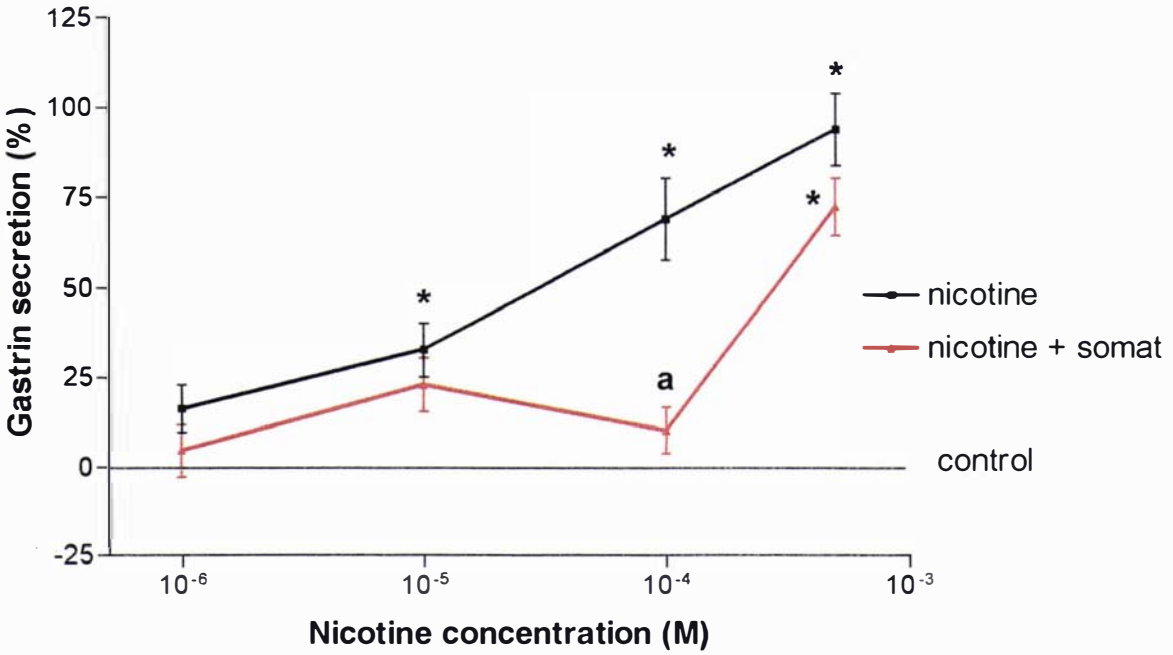

Figure 2.9. Effect of 10^{-6}M somatostatin on gastrin secretion stimulated by nicotine at concentrations from $5x10^{-4}$ to $1x10^{-6}$M. Data expressed as mean±SEM, *= response greater than control secretion, a= nicotine + somatostatin response less than the response to nicotine alone. These results are from two experiments showing similar response patterns.

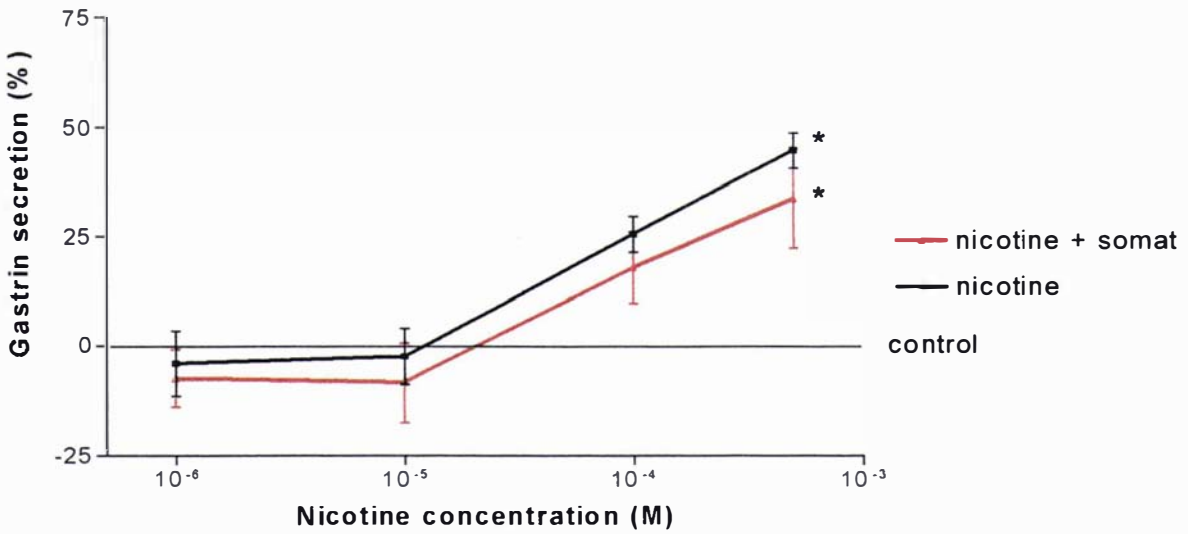

Figure 2.10. Effect of 10^{-6}M somatostatin on gastrin secretion stimulated by nicotine at concentrations from $5x10^{-4}$ to $1x10^{-6}$M. Data expressed as mean±SEM, *= response greater than control secretion. These results are from one experiment with significantly lower gastrin responses to nicotine than in experiments shown in Figure 2.9.

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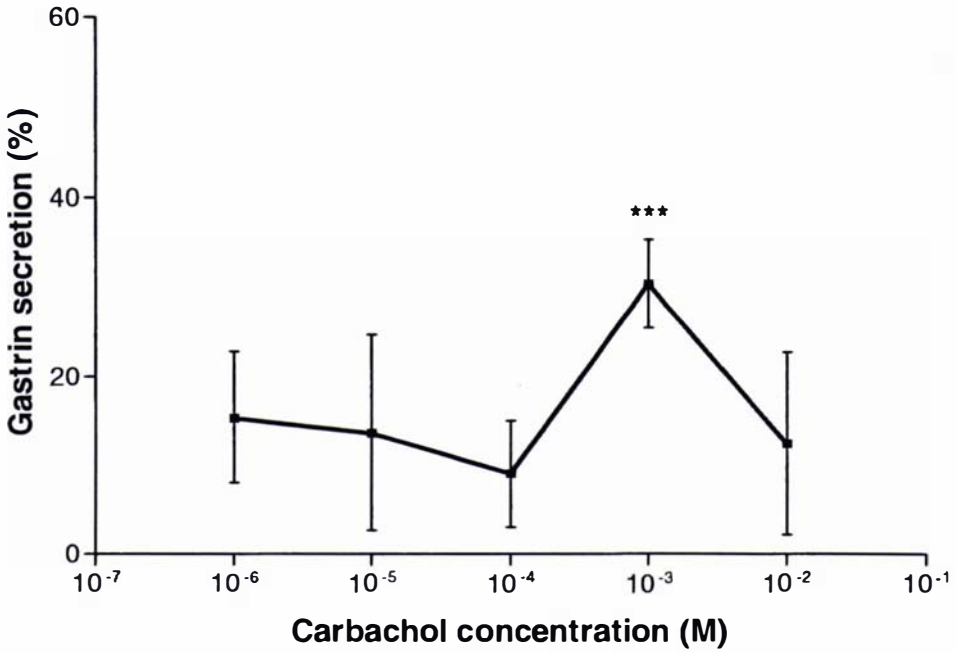

Figure 2.11. Effects of carbachol at concentrations from 10^{-2} to 10^{-6}M on gastrin secretion. Data expressed as mean±SEM, ***=p<0.001, secretion greater than control.

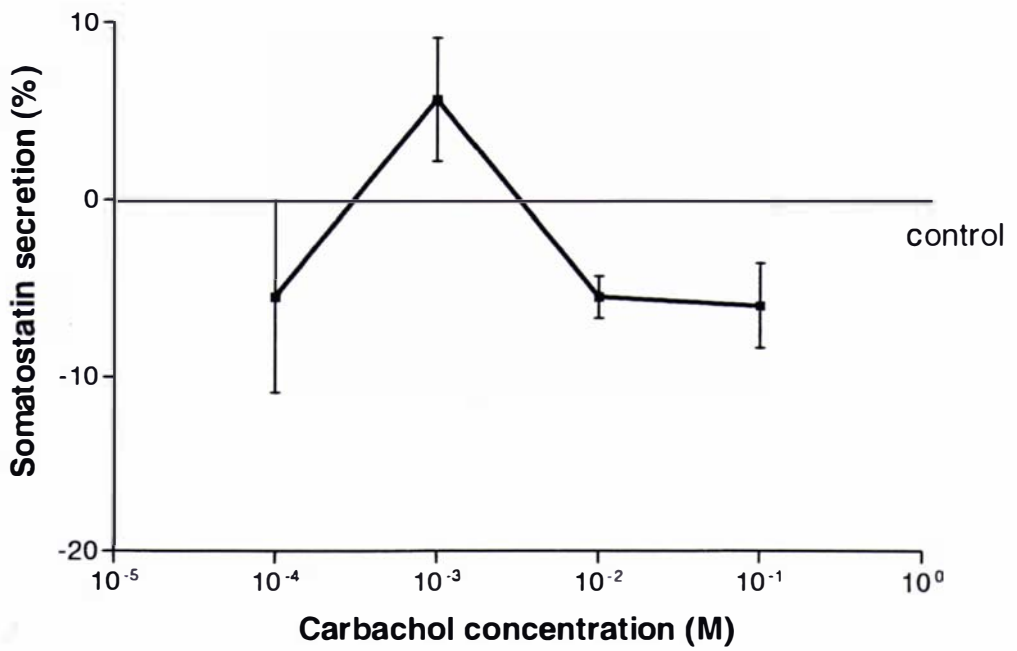

Figure 2.12. Effects of carbachol at concentrations from 10^{-2} to 10^{-6}M on somatostatin secretion. Data expressed as mean±SEM.

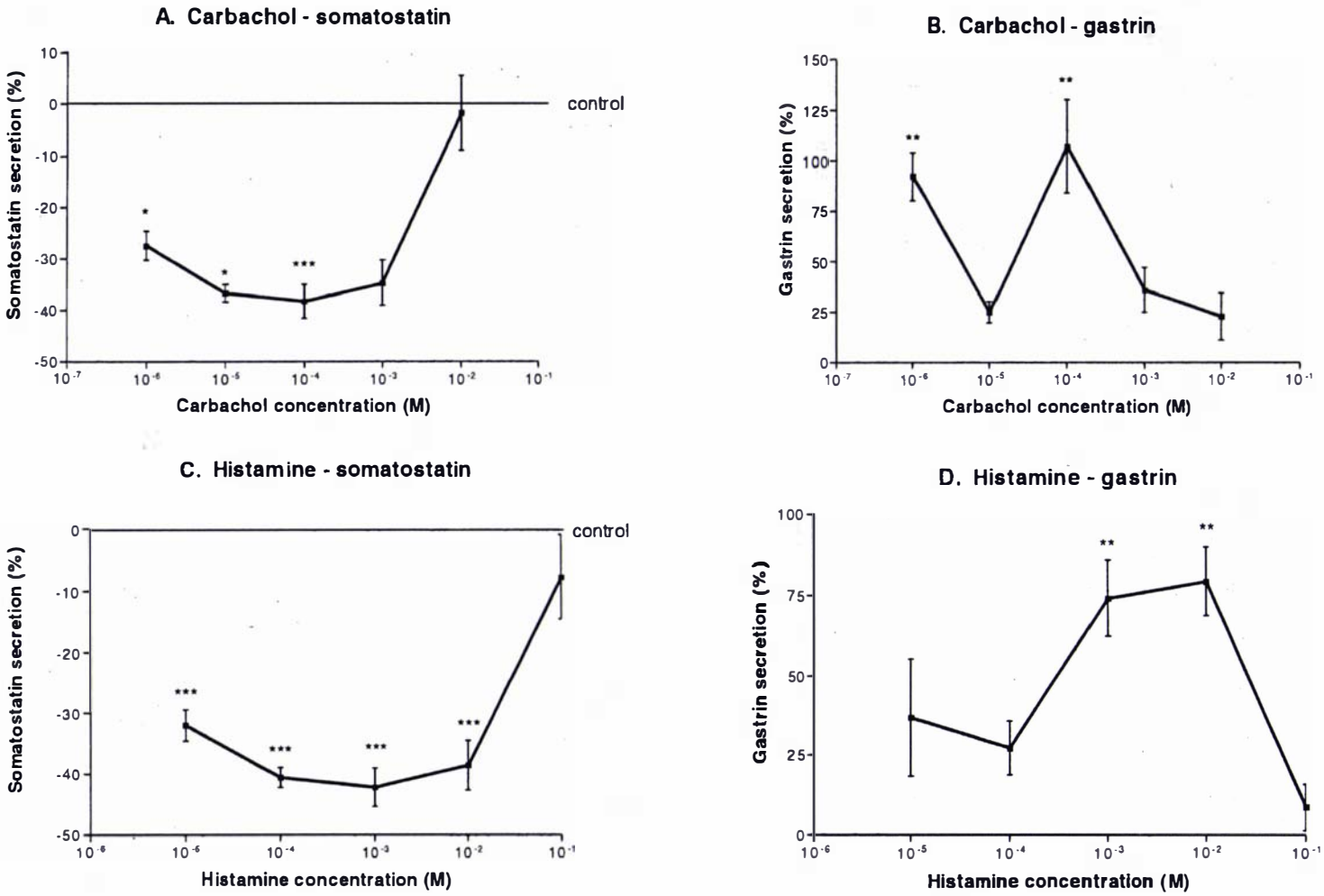

Figure 2.13. Effect of 10^{-5}M to 10^{-1}M carbachol or 10^{-5}M to 10^{-1}M histamine on somatostatin and gastrin secretion. Data expressed as mean±SEM. Graph A shows the effects of carbachol on somatostatin secretion, B carbachol on gastrin secretion, C histamine on somatostatin secretion and D histamine on gastrin secretion. Results different from control are marked, *=p<0.05, **=p<0.01, ***=p<0.001.

2.3.5 Somatostatin with Nicotine

Inclusion of somatostatin in the basal incubation medium at concentrations from 10^{-6}M to 10^{-9}M had no overall effect on basal gastrin release ($p>0.05$) (n=3), although there was variation in results between experiments ($p<0.001$). In one experiment, 10^{-7}M somatostatin caused a 40±4% reduction ($p<0.05$) in gastrin secretion, but no other significant effects were observed.

Three experiments tested the effect of 10^{-6}M somatostatin on nicotine-stimulated gastrin release. In two experiments for which the combined results are shown (Figure 2.9), somatostatin caused a reduction in gastrin secretion stimulated by nicotine only at 10^{-4}M. The gastrin secretion responses to nicotine and somatostatin were not ($p>0.05$) different between these two experiments. In the other experiment (Figure 2.10), only the highest concentration of $5x10^{-4}$M nicotine stimulated a significant increase in gastrin secretion of 39±9%, compared with nearly 100% increase in the other two experiments. Consistent with the other two experiments where it was tested, somatostatin did not affect the gastrin response at the $5x10^{-4}$M concentration.

2.3.6 Carbachol

Carbachol (n=4) had weak effects on gastrin release, stimulating secretion only at 10^{-3}M (Figure 2.11). The gastrin responses to 10^{-3}M carbachol varied between experiments ($p<0.05$) from 16±13% to 51±5%. Atropine (n=2) decreased 10^{-3}M carbachol-stimulated gastrin secretion, from +51±5% to +35±6% ($p<0.05$); similar results ($p>0.05$) were obtained from both experiments. Overall, carbachol did not affect somatostatin secretion ($p>0.05$) (Figure 2.12). In one experiment, however, carbachol inhibited somatostatin release at a concentration of 10^{-6}M, 10^{-5}M and 10^{-4}M, and gastrin secretion was markedly increased by 10^{-6}M and 10^{-4}M carbachol, where somatostatin inhibition had occurred (Figure 2.13A). The mean response to 10^{-3}M carbachol from all experiments was 30±5%, and was less than ($p<0.05$) the mean response to either pGRP (46±4%) or hGRP (43±5%).

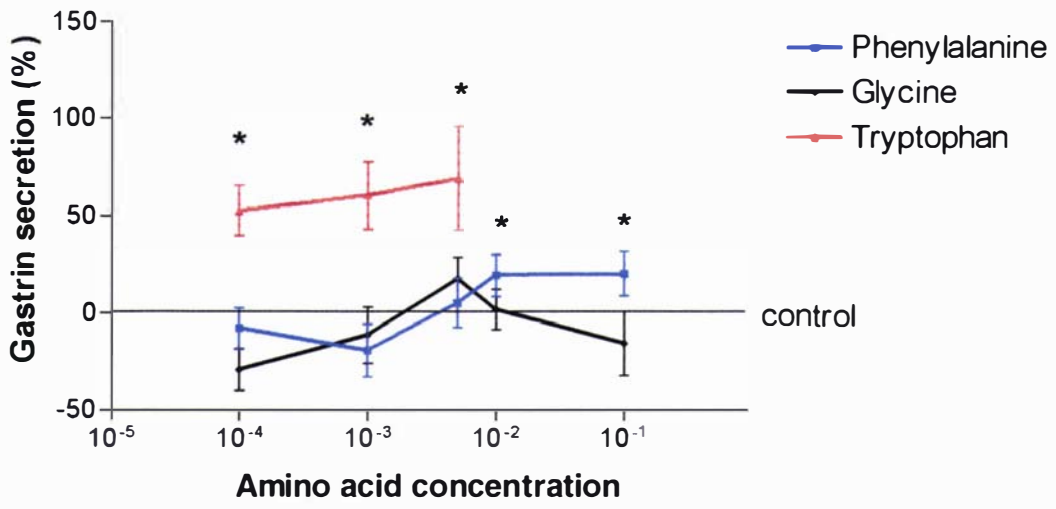

Figure 2.14. Effects of tryptophan, phenylalanine and glycine at concentrations of 10^{-4}M to 10^{-1}M on gastrin secretion. Data expressed as mean±SEM. Tryptophan at 10^{3}M, $5x10^{-3}$M and 10^{-2}M and phenylalanine at 10^{-2}M and 10^{-1}M stimulated gastrin secretion *=$p<0.05$

2.3.7 Histamine

Histamine at concentrations from 10^{-2} to 10^{-6}M had no effect on basal gastrin release ($p>0.05$). There was no variation between tissues in their responses to histamine ($p>0.05$) in two of the three experiments. Somatostatin secretion was also not significantly affected ($p>0.05$) by histamine in these two experiments. In the other experiment testing the effects of histamine, a decrease in somatostatin secretion was observed at all concentrations apart from 10^{-1}M histamine (Figure 2.13C), coinciding with an increase in gastrin secretion (Figure 2.13C). This experiment was the same as that described in Section 2.3.1.6, in which somatostatin secretion decreased in response to carbachol.

2.3.8 Adrenaline

Adrenaline at 10^{-4}M to 10^{-8}M had no significant effect on either somatostatin (-18±10% to +25 ±20%) or gastrin release (-5±8% to +5±6%).

2.3.9 Amino acids

Phenylalanine 10^{-2}M and 10^{-1}M stimulated gastrin release ($p<0.05$), with similar responses between experiments ($p>0.05$, Figure 2.14). Tryptophan at $5x10^{-3}$M, 10^{-3}M and 10^{-4}M consistently stimulated gastrin release by approximately 50% ($p<0.01$); the results did not differ between experiments ($p>0.05$) (Figure 2.14). When $5x10^{-3}$M of phenylalanine and $5x10^{-3}$M tryptophan were added together, gastrin secretion was increased, 30±6% ($p<0.05$), but this response was lower than that stimulated by tryptophan ($p<0.05$), and not different ($p>0.05$) from phenylalanine-stimulated gastrin release. Glycine had no significant effect ($p>0.05$) on gastrin secretion (Figure 2.14).

2.4 Discussion

The experiments described in this chapter showed that the basic pharmacology of gastrin and somatostatin secretion from unparasitised antral tissue is, in principle, similar to that reported by Lawton (1995), although tissue was less responsive to some agonists. The ganglionic stimulant nicotine was the most potent of all compounds tested in stimulating gastrin release. GRP was a more potent gastrin stimulant than carbachol whereas adrenaline had no effect.

In general, nicotine, GRP and carbachol had no significant effect on somatostatin secretion. Basal gastrin secretion appeared to be maximally inhibited by somatostatin, as neither VIP-stimulated somatostatin release nor somatostatin added to basal medium had any effect on basal gastrin secretion. However, VIP increased somatostatin and decreased gastrin secretion in the presence of GRP, while addition of somatostatin inhibited nicotine-stimulated gastrin release. Thus somatostatin is capable of inhibiting stimulated gastrin secretion.

The aromatic amino acids phenylalanine and tryptophan stimulated gastrin release. These results suggest that G cells may be responsive to luminal chemicals, such as amino acids *in vivo*.

2.4.1 Stimulatory responses

2.4.1.1 GRP

Both hGRP and pGRP were more potent than carbachol in stimulating gastrin release in experiments using unparasitised abomasal tissue. The greater potency of GRP in stimulating gastrin secretion is in agreement with results obtained by Lawton (1995) and *in vitro* experiments in rats (Richelson *et al.,* 1983; Gou *et al.,* 1987; Zeng *et al.,* 1996), dogs (Sugano *et al.,* 1987a) and humans (Campos *et al.,* 1989).

The observation that GRP had no significant effect on somatostatin secretion is in agreement with the results obtained by Lawton (1995) and research using human antral tissue (Richelson *et al.,* 1983; Campos *et al.,*

1989, 1990; Buchan *et al.,* 1990) or rat antral tissue (Richelson *et al.,* 1983). However, other research has shown that GRP stimulates somatostatin secretion in the perfused rat stomach (Duval *et al.,* 1981; Martindale *et al.,* 1982; Azuma *et al.,* 1987; Gou *et al.,* 1987; Schubert *et al.,* 1991) and pig antral tissue preparation (Holst *et al.,* 1987b, 1993). Stimulation of somatostatin release by GRP in the perfused rat stomach is reported to be via increased gastrin secretion (Schubert *et al.,* 1991), but stimulation of somatostatin release in pigs is thought to be via a direct action (Holst *et al.,* 1987b, 1993). Thus there appear to be species differences in the effect of GRP on antral somatostatin secretion.

The concentration of GRP necessary to stimulate gastrin release in the present experiments was greatly in excess of concentrations required for other species. Porcine antral gastrin secretion was doubled by 10^{-11}M GRP (Holst *et al.,* 1987a), with similar results for tissue from rats and humans (Richelson *et al.,* 1983). Sequence differences between ovine and human or porcine GRP may result in poorer binding of the latter two for the ovine GRP receptor. Fraser *et al.* (1994) reported that ovine GRP has an identical C-terminal decapeptide sequence to human and porcine forms, but has differences in the N-terminal sequence. Whether or not differences in the N-terminal are important for GRP receptor binding is unknown, but N-terminal differences do define differing receptor specificity for gastrin and cholecystokinin. Bombesin receptor subtype 3 (BRS-3) has been characterised in sheep (Whitley *et al.,* 1999) and shown to be different from BRS-3 in humans (Gorbulev *et al.,* 1994), mice and guinea pigs (Akeson *et al.,* 1997) at one amino acid site which is critical for agonist binding. BRS-3 has affinity for bombesin in the micromolar range, while other members of the GRP receptor family have affinity in the nanomolar range (Akeson *et al.,* 1997). Although BRS-3 may not be the receptor expressed in the sheep antrum causing the observed low affinity for GRP, the difference between sheep BRS-3 and BRS-3 in other species at a site critical for peptide binding illustrates that sheep GRP receptors may differ from those in other species, with different binding properties.

Results presented here, together with those of Lawton (1995), show that GRP consistently stimulates gastrin release *in vitro*, which is not the case *in*

vivo unless in the presence of somatostatin immunoblockade (Shulkes *et al.,* 1994; Zavros *et al.,* 1999). However, the percentage increase in gastrin levels over basal levels in response to 10^{-6}M GRP observed in the present experiments were as little as one third as big as those reported by Lawton (1995). In addition, in the experiments reported here, unlike those reported by Lawton (1995), the tissue was insensitive to 10^{-7}M GRP in both human and porcine forms. These discrepancies were unlikely to have been due to the experimental method, which was identical to that used by Lawton (1995). The degree of abomasal parasitism may have been a source of variation. All tissues used for experiments presented here were from parasite-free abomasa. The abomasal parasite status of sheep tissues used by Lawton (1995) was unknown, but as these animals came from pasture, without the presence of a parasite control regimen, all animals must be assumed to have been parasitised. The inflammation and gastritis that may accompany abomasal parasitic infection could increase the sensitivity of the G cell. In humans, *H. pylori* infection increases the sensitivity of the G cell to stimulation by GRP (Graham *et al.,* 1991; Beardshall *et al.,* 1992; Gibbons *et al.,* 1997).

2.4.1.2 Carbachol

Carbachol stimulated gastrin release (Figure 2.14) as in the experiments of Lawton (1995) and *in vitro* preparations from other species (Harty and McGuigan, 1980; Richelson *et al.,* 1983; Wolfe *et al.,* 1983; Sue *et al.,* 1985; Zaki *et al.,* 1996). Stimulation of gastrin secretion with carbachol occurred only at 10^{-3}M concentration, and was less than the mean response caused by either human or porcine GRP, though the differences between GRP and carbachol stimulated gastrin release were not as large as those reported by Lawton (1995). As with the GRP, the tissue used for the present experiments was less responsive to carbachol than that used by Lawton (1995), requiring greater concentrations to produce smaller effects.

Carbachol stimulates gastrin release via M_3 receptors on canine G cells (Yokotani *et al.,* 1995). It is therefore possible that the muscarinic actions of carbachol on gastrin secretion in experiments described here were via direct stimulation of the G cell.

It was unclear whether the action of carbachol was via muscarinic or nicotinic receptors in the experiments of Lawton (1995). Although atropine abolished the gastrin response to carbachol, bethanechol did not stimulate gastrin release. Furthermore, whereas nicotine stimulated gastrin release, hexamethonium tended to enhance carbachol induced gastrin release (Lawton, 1995). In the experiments presented here, atropine partially inhibited carbachol stimulation of gastrin release, indicating carbachol acted via both nicotinic and muscarinic mechanisms. The greater concentrations of carbachol used in these experiments may be responsible for the lesser effects of atropine compared with those observed by Lawton (1995). Decreased responsiveness and the reduced effect of atropine suggests that the muscarinic stimulation of gastrin release is much less in these experiments than in those conducted by Lawton (1995).

It has been widely reported that carbachol stimulates gastrin release indirectly by inhibiting somatostatin release (Harty and McGuigan, 1980; Schubert e*t al.,* 1982; Richelson e*t al.,* 1983; Wolfe e*t al.,* 1983; Sue e*t al.,* 1985). This was also demonstrated in sheep *in vivo* (Zavros e*t al.,* 1999). Lawton (1995) noted variability in the somatostatin response to carbachol: in four cases somatostatin was stimulated, while in another two it was inhibited. Overall, there was no change in somatostatin secretion in response to carbachol in the present experiments (Figure. 2.12). This suggests that the observed stimulation of gastrin secretion was via a direct effect on the G cell and not via reduced somatostatin secretion.

2.4.1.3 Nicotine

Nicotine was the most effective stimulant of gastrin release in this experimental preparation, but had no overall effect on somatostatin secretion. This suggests that the dominant effect of nicotinic agonism is stimulation of the G cell. The effect of nicotine is most likely due to a general stimulation of postganglionic neurons. However, a direct effect on the G cell cannot be ruled out, as the exact site of action of nicotine in the present experiments is uncertain. Although not examined histologically, it is possible the neurons from the submucosa were not removed during tissue dissection, and that nicotine acts on these neurons. Stimulation of gastrin secretion by nicotine is in

agreement with results in the perfused rat stomach (Schubert and Makhlouf, 1982; Schubert *et al.,* 1985) and in an antral mucosal preparation (Schubert and Makhlouf, 1987) in which the nicotinic agonist 1,2 dimethyl-4-phenylpiperazinium (DMPP) increased gastrin secretion.

Gastrin release stimulated by nicotine in the present experiments was well in excess of the sum of the mean GRP and carbachol stimulatory effects. It is possible that this was due to the GRP responses being submaximal as a result of poor stimulation of the sheep GRP receptor by porcine and human GRP. In the perfused rat stomach, over 90% of the gastrin response to DMPP was inhibited by a combination of atropine and bombesin antiserum (Schubert *et al.,* 1985), and in a rat antral preparation, stimulation of gastrin secretion was predominantly via noncholinergic mechanisms (Schubert and Makhlouf, 1987). If gastrin secretion stimulated release from ovine antral tissue is similar to that in rat antral tissue, it seems likely that poor stimulation of gastrin release from ovine G cells, using hGRP and pGRP, is responsible for the discrepancy between the combined GRP and carbachol results and the nicotine response. The discrepancy could also be due to the presence of another gastrin stimulatory compound. However, adrenergic effects are not responsible, as adrenaline had no effect on gastrin or somatostatin secretion in these experiments, or those of Lawton (1995).

Lawton (1995), reported that the gastrin response to nicotine was less than that to porcine GRP, which is at variance with these results. This may be due to a greater degree of removal of nicotinic-sensitive neurons in the dissection of the tissue in the experiments of Lawton (1995) compared with those described here.

2.4.1.4 Amino acids

Amino acids stimulated gastrin release in the present experiments. Stimulation of gastrin release by amino acids has been reported in dogs (Konturek *et al.,* 1977b), rats (Lichtenberger *et al.,* 1982a; Dial *et al.,* 1991) and humans (Taylor *et al.,* 1982). Taylor *et al.* (1982) showed that the aromatic amino acids phenylalanine and tryptophan were the most potent in stimulating gastrin secretion in humans. Both were effective in stimulating gastrin release in the experiments presented here. DelValle *et al.* (1990) reported that the

effects of amino acids were via a receptor-mediated action. Another possibility is that amino acids stimulate gastrin release by destabilising the gastrin granule in a similar manner to basic amines and decarboxylated amino acids (Dial *et al.,* 1991). This mode of action would favour hydrophobic amino acids. Interestingly, on the one occasion where phenylalanine and tryptophan were used in combination, the gastrin response was different to the gastrin response initiated by phenylalanine or tryptophan alone. The mixed solution produced a response less than that of tryptophan alone. Although direct comparison of the phenylalanine and mixed solution responses revealed no significant differences, the response to the mixed solution was significantly increased over basal levels, while the response to phenylalanine alone was not. Thus it appears that the gastrin response to the mixed solution was somewhere between the responses to phenylalanine and tryptophan alone. This suggests a competitive, rather than additive, relationship between phenylalanine and tryptophan in the stimulation of gastrin release.

2.4.2 Gastrin inhibitors

Study of the inhibition of gastrin release in experiments presented here focused on the effects of somatostatin. The experiments conducted tested whether the stimulation of somatostatin release affected basal or stimulated gastrin release, and whether inclusion of somatostatin in the basal medium at very high concentrations could affect basal or stimulated somatostatin release.

2.4.2.1 Effects of somatostatin on basal gastrin secretion

VIP increased somatostatin secretion in experiments presented here, as it did in the perfused rat stomach (Chiba *et al.,* 1980a; Saffouri *et al.,* 1984a), the porcine antrum (Holst *et al.,* 1993) and in sheep antral tissue (Lawton, 1995), but did not inhibit basal gastrin levels as in the perfused rat stomach (Saffouri *et al.,* 1984a) and sheep antral tissue (Lawton, 1995). Somatostatin responses to VIP were less than those reported by Lawton (1995).

GIP did not stimulate somatostatin release, in contrast to observations by Lawton (1995) or results from experiments in pigs (Holst *et al.,* 1983) and rats (McIntosh *et al.,* 1984; Wolfe *et al.,* 1986). At times, there appeared to be a tendency for GIP to increase gastrin secretion, but the response was never

significant. McIntosh *et al.* (1984) also reported that GIP occasionally stimulated gastrin in rats.

Addition of somatostatin to the incubation medium, even at micromolar concentrations, did not inhibit basal gastrin secretion. Reports of the effects of somatostatin from other species are inconsistent. Increased somatostatin levels did not affect basal gastrin secretion in some *in vitro* preparations of rat (Hayes *et al.,* 1975) and human tissue (Campos *et al.,* 1990), but did decrease gastrin release in other preparations of rat tissue (Zaki *et al.,* 1996), and with canine (Zaki *et al.,* 1996) and porcine (Holst *et al.,* 1992) tissue. This variation in results may be due to differences in the *in vitro* preparation techniques. In sheep, gastrin is thought to be under a high level of somatostatin restraint *in vivo* (Shulkes *et al.,* 1994; Zavros *et al.,* 1999).

In general, nicotine did not affect somatostatin secretion in experiments presented here, in contrast to results from the perfused rat stomach (Schubert and Makhlouf, 1982; Schubert *et al.,* 1985) and from rat antral mucosal preparations (Schubert and Makhlouf, 1987), in which the DMPP increased somatostatin secretion. The stimulation of somatostatin secretion by DMPP in the rat stomach is a GRP-mediated action (Schubert *et al.,* 1985) and is atropine resistant in antral preparations (Schubert *et al.,* 1987). It has subsequently been shown that GRP stimulation of somatostatin release in the rat antrum is via stimulation of gastrin release (Schubert *et al.,* 1991), but in the pig is via direct action on the D cell (Holst *et al.,* 1987b, 1993). In general, GRP was unable to stimulate somatostatin release in experiments presented here, which may be why nicotine failed to stimulate somatostatin release.

It is also possible that the lack of effect of nicotine on somatostatin secretion is due to an inhibitory neural effect masking stimulatory factors. The lack of a significant effect on somatostatin secretion by nicotine is most likely due to a summation of many neural factors, rather than one specific action. Even if GRP does not stimulate antral somatostatin secretion in sheep, there are stimulatory neural pathways to the ovine antral D cell, as the neuropeptide VIP can stimulate somatostatin release. If these stimulatory neurons were activated by nicotine, their effects appear to have been cancelled out by inhibitory effects on the D cell.

None of the pharmacological agents tested in experiments presented here consistently inhibited somatostatin secretion. Notably, carbachol and histamine were unable to inhibit somatostatin secretion unlike in rat antral tissue where cholinergic effects (Richelson *et al.,* 1983; Wolfe *et al.,* 1983; Sue *et al.,* 1985; Schubert *et al.,* 1987) and histamine (Vuyyuru *et al.,* 1995) inhibited somatostatin secretion. However, in two experiments, basal somatostatin was inhibited by pharmacological agents. In one of these, nicotine and GRP inhibited somatostatin secretion, while in the other carbachol and histamine inhibited basal somatostatin release. A common feature of these two experiments was that suppression of somatostatin secretion in the presence of agonists resulted in a greater increase in gastrin secretion than in experiments where the same agonists were used, but somatostatin was unaffected. This was most obvious in the experiment in which carbachol and histamine suppressed somatostatin secretion (see Figure 2.13). In this experiment, reduction of somatostatin secretion increased the sensitivity of the G cell to carbachol such that lower doses would stimulate gastrin release. This was also the only experiment in which histamine reduced somatostatin secretion and increased gastrin secretion, as has been reported in rats (Bado *et al.,* 1994; Vuyyuru *et al.,* 1995; Schubert and Makhlouf, 1996). It is unclear why nicotine, GRP, histamine or carbachol were only able to suppress somatostatin release in two experiments and not others where the same pharmacological agents were used. There was no difference in experimental conditions, pharmacological agents (in batch number or preparation) and basal somatostatin release was in the middle range in these experiments when compared with concentrations observed in other experiments (Table 2.1).

2.4.2.2 Effects on stimulated gastrin secretion

VIP reduced GRP-stimulated gastrin release and increased somatostatin secretion. It was unclear whether this was due to a direct action of VIP on the G cell or an effect mediated through increased somatostatin levels. Somatostatin at micromolar amounts was able to reduce nicotine-stimulated gastrin release, such that gastrin secretion was not significantly elevated above control levels by 10^{-5}M and 10^{-4}M nicotine solutions. This suggests that

somatostatin does indeed have direct effects which inhibit gastrin secretion from the G cell. Interestingly, nicotine was not able to inhibit maximal gastrin secretion caused by nicotine at $5x10^{-4}$M concentration. This suggests that the G cell has a point of maximal stimulation at which somatostatin will not inhibit secretion.

2.5 SUMMARY

The pharmacology of the ovine antral G cell is similar to that observed in monogastric animals. Results from experiments presented in this Chapter showed that gastrin was most potently stimulated by nicotine (maximum response +118±7%), followed by human (+43±5%) or porcine (+46±4%) GRP and carbachol (+30±5%), with adrenaline having no significant effect. It is suggested that GRP is the main stimulant of gastrin release in sheep, although the ovine GRP receptor has different binding properties to the human and porcine GRP receptor. Gastrin secretion was also increased by the aromatic amino acids phenylalanine (+32±10%) and tryptophan (+56±16%), suggesting the G cell is sensitive to luminal chemicals.

Somatostatin even at very high concentration in the incubation medium, could not inhibit basal gastrin release. However, VIP-stimulated somatostatin release could inhibit GRP-stimulated gastrin release (reducing the GRP-stimulated gastrin response from +61±8% to +18±7%). The inclusion of somatostatin in basal medium did reduce the gastrin response to some concentrations of nicotine (abolishing the stimulation of gastrin release by 10^{-5}M and 10^{-4}M nicotine). Thus, somatostatin can inhibit gastrin secretion, but basal gastrin secretion is maximally restrained by somatostatin. The antral G cell may be more sensitive to stimulants when the abomasum is subject to parasitism, in a similar fashion to the G cell in monogastric animals which is more sensitive during gastritis.

CHAPTER 3
HYPERGASTRINAEMIA DURING *OSTERTAGIA CIRCUMCINCTA* INFECTION: CAUSES AND EFFECTS

3.1 Introduction

Hypergastrinaemia, hyperpepsinogenaemia and hypochlorhydria are features of parasitism of sheep by *O. circumcincta* (Armour *et al.,* 1966; McLeay *et al.,* 1973; Anderson *et al.,* 1976; Lawton *et al.,* 1996) or *H. contortus* (Christie *et al.,* 1970; Fox *et al.,* 1988; Nicholls *et al.,* 1988; Simpson *et al.,* 1997) and of cattle by *O. ostertagi* (Jennings *et al.,* 1966; McKellar *et al.,* 1986; Snider *et al.,* 1988; Fox *et al.,* 1993).

Elevated abomasal pH has been proposed as the principal cause of the hypergastrinaemia. The serum gastrin concentration initially rises with abomasal pH (Fox *et al.,* 1988a, 1993; Lawton *et al.,* 1996), presumably due to lack of negative feedback by acid (Anderson *et al.,* 1981; Fox *et al.,* 1988a, 1993; Lawton *et al.,* 1996). Thereafter, patterns of gastrin secretion appear to diverge between experiments. In calves infected with *O. ostertagi* there is a strong correlation between abomasal pH and serum gastrin (Fox *et al.,* 1993). In sheep, however, while abomasal pH and serum gastrin increase together, serum gastrin may remain elevated after abomasal pH decreases, (Lawton *et al.,* 1996). Furthermore, in four sheep given 150 000 exsheathed L3 *O. circumcincta* larvae, Lawton *et al.* (1996) reported that serum gastrin decreased to pre-infection levels during periods of very high abomasal pH (over pH5.5), with hypergastrinaemia again occurring when abomasal pH began to decrease, and in some cases remained after abomasal pH returned to normal. While the results in calves suggest fluctuations in abomasal pH can explain hypergastrinaemia in cattle, the results in sheep appear more complex. It was postulated that a luminal factor interferes with gastrin secretion at very high abomasal pH levels, while another factor elevates gastrin levels when abomasal pH has returned to normal ranges (Lawton *et al.,* 1996).

On the basis of *in vitro* evidence of bacterial inhibition of gastrin release (Haag, 1995; Lawton, 1995), it was suggested that this decrease in circulating gastrin may be associated with the increased microbial survival in the less acidic abomasum (Lawton *et al.,* 1996). Numbers of both aerotolerant (Jennings *et al.,* 1966) and anaerobic bacteria (Nicholls *et al.,* 1987) have been reported to increase during abomasal parasitism, but serum gastrin concentrations were not compared with changes in the bacterial populations. In calves infected with *O. ostertagi*, increased aerotolerant bacterial numbers in abomasal contents were coincident with the onset of diarrhoea (Jennings *et al.,* 1966). Higher numbers of abomasal anaerobes, coincident with increased pH and decreased oxygen tension of the abomasal contents, were reported in sheep after infection with a single, extreme dose of one million *H. contortus* larvae (Nicholls *et al.,* 1987).

The increase in circulating concentrations of gastrin has been postulated to be either beneficial to the host, aiding recovery from infection through stimulating replacement of lost parietal cells and inducing hypersecretion from those remaining (Scott *et al.,* 1998a), or detrimental by causing reduced gut motility and anorexia (Fox *et al.,* 1989a, b; Fox, 1997).

In this chapter, two *in vivo* experiments are described. The aims of the first experiment (LPI-1) were: to determine whether bacterial numbers do increase in the abomasum during *O. circumcincta* infection and if so whether this increase affects serum gastrin levels; to determine what changes in abomasal conditions facilitate increased bacterial survival; to determine whether hypergastrinaemia correlates with increased abomasal pH and whether hypergastrinaemia correlates with decreased food intake as it does in calves infected with *O. ostertagi.* In the second experiment (LTI) the aim was to determine oxygen concentrations in the abomasum and whether these changed during parasitism.

3.2 Materials and methods

3.2.1 Experiment 1 (LPI-1)

3.2.1.1 Animals

Five, six month-old female Romney cross lambs (30-35kg body weight) had been surgically fitted at 12 weeks-of-age with abomasal cannulae at the junction of the antrum and the fundus under general anaesthesia, as previously described by Lawton *et al.* (1996). Three of the lambs had been raised nematode-free until infection (Sheep 1-3) and two (Sheep 4-5) had been infected with *T. colubriformis* at four months-of-age and the infection terminated after 21 days by dosing with 8mg.kg^{-1} levamisole HCl (Rycozole, Young's Animal Health) (two weeks prior to commencement of the present experiment). There was no evidence of abomasal involvement in the earlier parasitism (abomasal pH and serum gastrin and pepsinogen concentrations were unchanged). No eggs were detected in the faeces of any of the five animals 10 days prior to infection.

After a control sampling period of six days (Days 1-6), each sheep received 150 000 exsheathed *O. circumcincta* larvae via the abomasal cannula on Day 7 and a further 100 000 sheathed L3 were administered into the rumen by tube on Day 18. The sheep were housed in individual metabolism crates and provided with 2kg of pelleted lucerne daily to allow *ad libitum* feeding. Actual daily feed intake was measured. Water was freely available.

3.2.1.2 Blood and abomasal fluid samples

Blood and abomasal contents were collected daily prior to and/or three hours after feeding. Abomasal fluid was also collected for bacterial estimations twice daily on Days 1 and 5 prior to infection, and on Days 7, 13, 15, 18, and once daily on Days 22, 29 and 42 after infection. When sampling occurred twice daily, the data from each sample were treated as separate data points. Blood was collected by jugular venepuncture into plain vacuated tubes, centrifuged at 2000*g* for 20 minutes after collection, the serum separated and stored at -20°C for gastrin and pepsinogen assay. Abomasal contents were routinely collected by spontaneous flow from the opened cannulae into tubes which were

centrifuged at 2000*g* for 30 minutes and the supernatant used to measure pH with a PHM82 Standard pH Meter (Radiometer) and for pepsinogen estimation. For bacterial estimations, abomasal fluid was collected into sterile bottles gassed with CO_2 for anaerobic culture or containing air for aerotolerant culture.

3.2.1.3 Serum gastrin

Serum gastrin was determined in triplicate by a radioimmunoassay, as described in Appendix 1.

3.2.1.4 Serum and abomasal pepsinogen

Abomasal peptic activity and serum pepsinogen concentration were determined using a method previously validated by Scott *et al.* (1995) and described in Appendix 3.

3.2.1.5 Aerotolerant bacteria

Aerotolerant bacteria were enumerated aerobically on agar (1.5% w/v) Bacto Agar Brain Heart Infusion medium (Difco Laboratories, USA). Abomasal fluid (0.1mL) was transferred by sterile pipette from sterile air-filled collection bottles to sterile glass tubes containing 0.154M isotonic saline (0.9mL) for decimal dilution. Relevant dilutions were transferred by pipette to plates and incubated for 48 hours at 37°C. Plates containing 3-30 independent colonies were scored. All dilutions were conducted in duplicate and the mean count from all plates within the scoring criteria was taken as the number of viable aerotolerant bacteria. Abomasal fluid samples were processed within an hour of collection.

3.2.1.6 Anaerobic bacteria

The methods used for the preparation of pre-reduced medium and anaerobic culture techniques were those described by Hungate (1969). Enumerations were carried out in 16mL screw-cap Hungate tubes (Bellco Glass Inc.) containing 6.3mL BYC medium under an O_2-free CO_2 headspace. BYC medium consisted of 30% rumen fluid medium of Joblin *et al.* (1990) supplemented with yeast extract ($2g.L^{-1}$), glucose ($0.5g.L^{-1}$), cellobiose ($0.5g.L^{-1}$), xylose ($0.5g.L^{-1}$) and maltose ($0.5g.L^{-1}$). The anaerobic vitamin solution (0.07mL) of Balch *et al.* (1979) was added to each tube after

autoclaving. Abomasal fluid samples were processed within an hour of collection. For decimal dilutions, the initial anaerobic transfer (0.7mL) into BYC medium was carried out by pipette, because of particulate matter in the abomasal fluid. All subsequent dilution transfers were by syringe. Tubes were incubated at 39°C and the highest dilutions showing growth (turbidity) after four days were scored as positive, to give counts to the nearest decimal level. Each dilution series was carried out in duplicate and the mean count from the two series was taken as the minimal viable anaerobic bacterial count.

3.2.1.7 Parasitology

Infective larvae (L3) were obtained from cultures of faeces from sheep infected with a pure strain of *O. circumcincta*. Motility was confirmed before infection. Larvae were exsheathed with 0.2% sodium hypochlorite and rinsed with water one hour before abomasal infection. Egg counts (e.p.g.) using a modified McMaster method (Stafford *et al.,* 1994) were performed on faeces collected *per rectum* 14 and three days before infection and 25, 29, 32 and 36 days post-infection. Worms were counted in abomasal contents and in pepsin-digested tissue collected at necropsy.

3.2.1.8 Faecal consistency

Faecal consistency was scored on a scale of 1 to 4. 1 indicated pellets, 2 pellets adhering to each other, 3 unpelleted but solid faeces and 4 fluid faeces. Faecal consistency was monitored daily.

3.2.1.9 Histology

At necropsy, the abomasum was gently washed and dabbed dry with tissue paper, then three 1cm diameter tissue samples were taken from the antrum, avoiding nodular tissue where possible. Samples were fixed by immersion in Bouin's fluid for six to eight hours, dehydrated in 70% alcohol, then embedded in paraffin using an automatic tissue processor (SE400, Shandon Scientific Co.) and sections 6μm thick were cut. Sections were then rehydrated using xylene and alcohol.

3.2.1.9.1 G cell staining

Antral sections were incubated with the following antibodies (dissolved in Phosphate Buffered Saline [PBS], 1% BSA): gastrin antiserum (1:200 rabbit anti-human, Amersham International) for 1 hour, second antibody (1:200 Donkey anti-rabbit, Amersham International) for 30 minutes, then third antibody (1:200 Streptavidin-biotinylated horseradish peroxidase complex, Amersham International) for 15 minutes. Sections were then counterstained with Hematoxylin. For each animal, G cell numbers in glands sectioned longitudinally were counted in six fields at 40x magnification for each animal.

3.2.1.9.2 Parietal cell staining

Fundic sections were incubated with the following antibodies (dissolved in PBS, 1% BSA): TGFα antiserum (Oncogene Research Products, USA) for one hour, second antibody (1:200 sheep anti-mouse Amersham International) for 30 minutes, then third antibody (1:200 Streptavidin-biotinylated horseradish peroxidase complex, Amersham International) for 15 minutes. Sections were then counterstained with Hematoxylin. Parietal cells were then counted in three fields per animal. For each animal, parietal cell numbers were counted in glands sectioned longitudinally at 40x magnification in 258μm columns in six fields and the depth of tissue recorded.

3.2.1.10 Statistical analyses

For abomasal pH, serum gastrin and pepsinogen, the upper limit of the normal range was established for each animal and for the group as two standard deviations (SD) above the mean of all pre-infection values. A lower limit of two SD below the mean was similarly calculated for food intake. Statistical tests were conducted using SPSS version 9.0. Paired t-tests were used to compare morning and afternoon control samples for each animal for abomasal pH and serum pepsinogen and gastrin concentrations. As the distribution of pre-infection data for serum gastrin and abomasal pH were significantly skewed, Spearman correlations were performed, one-tailed for serum gastrin versus abomasal pH and versus food intake, but otherwise were two-tailed. Using NCSS 97, piecewise polynomial regression was used to model the relationship between anaerobic bacterial counts and abomasal pH. G cell

numbers, parietal cell numbers and fundic mucosal depth were compared using one-way ANOVA. Data sets in comparisons were tested for equality of variance using Levene's Homogeneity of Variation test. *Post hoc* comparisons of data groups were conducted using Tukey's HSD test for comparisons of all groups.

3.2.2 Experiment 2 (LTI)

3.2.2.1 Animals

The samples for this experiment were obtained from sheep in an experiment by Ms. Sabine Przemeck.

Eight, six month-old Romney cross lambs (30-35kg body weight) were raised parasite-free and surgically fitted with abomasal cannulae as previously described in Section 3.2.2.1. After two weeks, control sampling began six days prior to infection. Sheep were infected with 50 000 L3 *O. circumcincta* on Day 0, followed by 10 000 larvae once a week for six weeks (on Days 35, 42, 49, 56, 63 and 70). Abomasal samples for oxygen estimation were taken from sheep on Days 5, 6 and 7 after the first infection, after the third trickle infection on Days 51 and 54, after the last trickle infection on Days 78, 84 and 90. Samples were collected from seven sheep on the first four sampling days and from four sheep on the subsequent five occasions.

3.2.2.2 Measurement of oxygen concentration

Samples were collected by opening the abomasal cannula, allowing contents to flow out for two seconds, then placing a 50mL centrifuge tube on the still open cannula, collecting abomasal contents and then sealing the tube when it was filled. The sample was then poured from the tube into a closed stirred cell maintained at 37°C and the oxygen concentration measured. The time between collection of sample and estimation of oxygen concentration was less than one minute. The oxygen electrode used was maintained at 37°C and calibrated against aerated H_2O, which at this temperature contains 240μM O_2.

On each of the last two days of sampling, a second sample was taken from each of four sheep and, in addition to the initial measurement as described above, oxygen concentration of the sample measured after standing for 30

minutes in a sealed tube and in supernatant after centrifugation of the sample for 30 minutes at 2000*g*.

Further, the stability of the oxygen concentration in abomasal samples, which had been gassed for five minutes with 100% oxygen was tested. Samples were placed in the sealed oxygen measurement cell for 40 minutes and oxygen concentration recorded every five minutes. For these measurements, the oxygen electrode was calibrated with oxygen saturated H_2O (containing 1.2mM O_2), prepared by gassing for five minutes with 100% oxygen. The rate of oxygen decline in solutions was compared with the decline in oxygenated H_2O over the same time period, and with oxygenated abomasal contents to which 5% sodium hypochlorite (v/v) was added.

3.2.2.3 Abomasal pH

Abomasal contents for pH estimations were routinely collected as described in Section 3.2.2.2. The pH was measured with a PHM82 Standard pH Meter (Radiometer).

3.2.2.4 Statistical analyses

Results were analysed using SPSS version 9.0. All data were examined for normality using Shapiro-Wilks tests. The effect of centrifugation on oxygen concentration, difference in oxygen centrifugation between animals and between days were all analysed using one-way ANOVA. Data sets in comparisons were tested for equality of variation using Levene's Homogeneity of Variance test. *Post hoc* comparisons of data groups were conducted using Dunnett's *post hoc* test, for effects of centrifugation and Tukey's HSD for other tests.

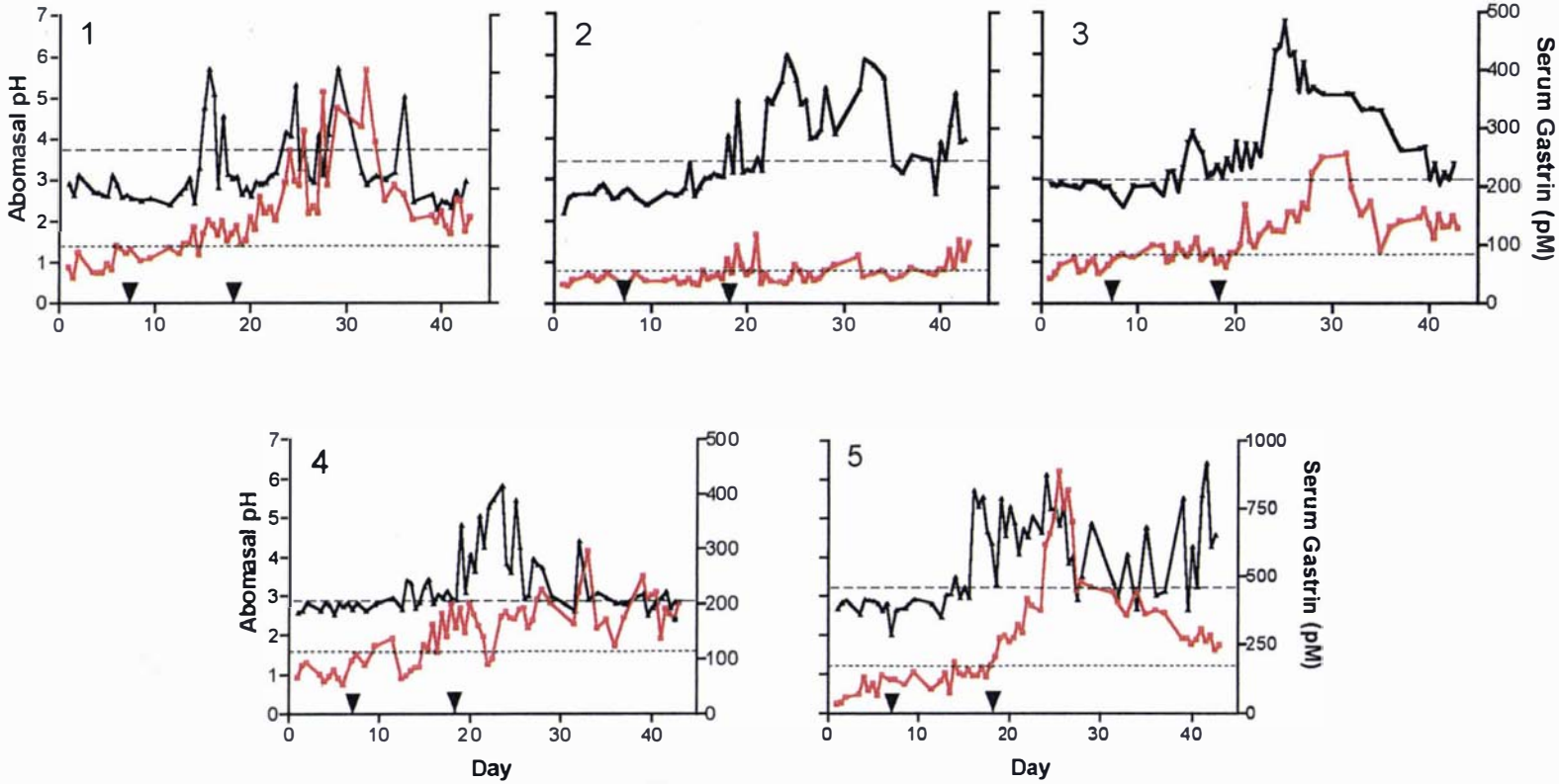

Figure 3.1. Serum gastrin (—) and abomasal pH (—) for Sheep 1-5 infected on Day 7 with 150 000 exsheathed L3 *O. circumcincta* and Day 18 with 100 000 sheathed L3 *O. circumcincta*. Infection times are indicated with arrowheads. The upper horizontal dotted lines represent 2 SD above the mean values of abomasal pH before infection, and the lower dotted lines 2 SD above the mean values for serum gastrin before infection.

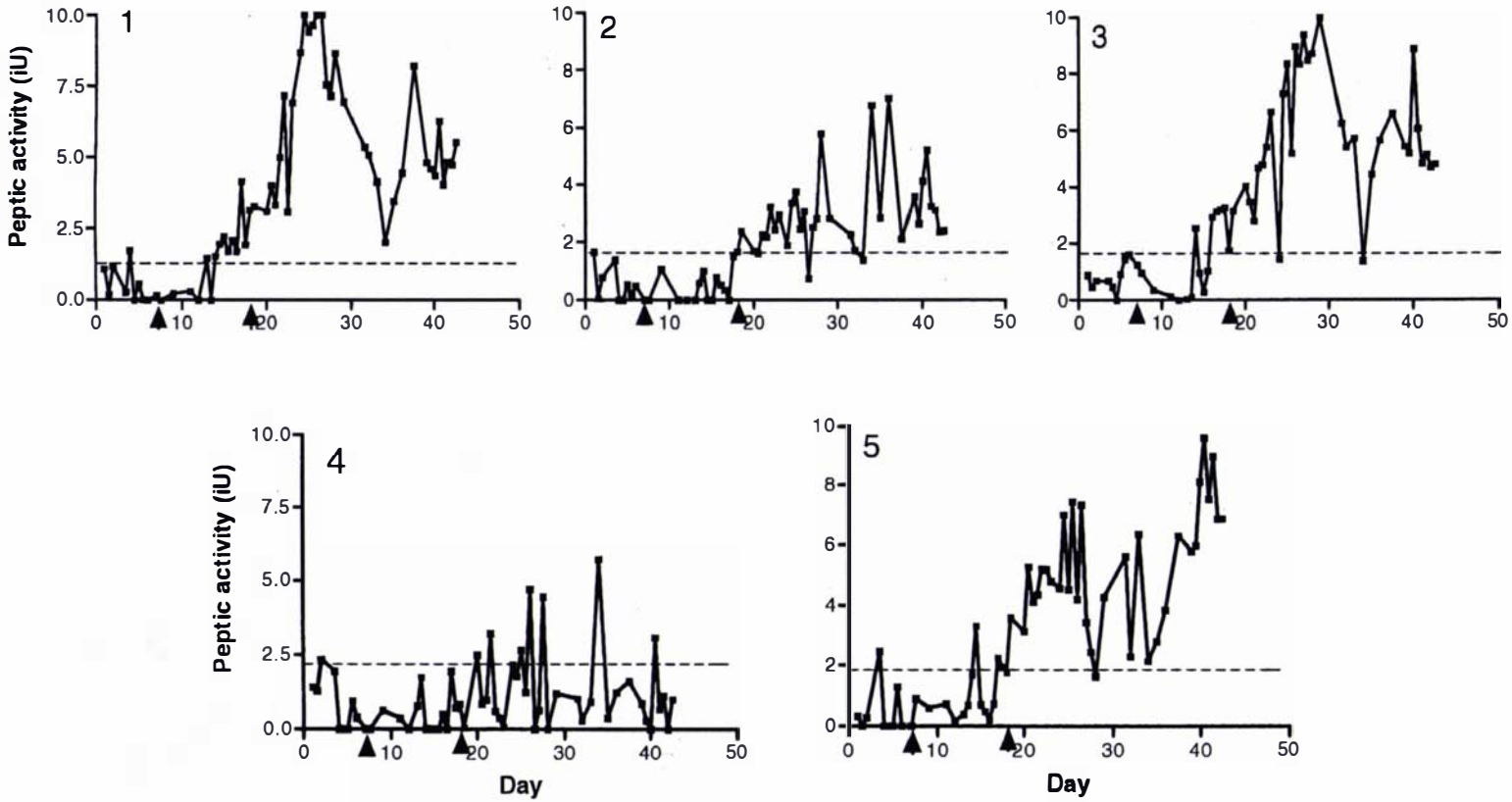

Figure 3.2. Serum pepsinogen for Sheep 1-5 infected on Day 7 with 150 000 exsheathed L3 *O. circumcincta* and Day 18 with 100 000 sheathed L3 *O. circumcincta.* Infection times are indicated by arrowheads, with dotted line showing 2.SD above the mean values for serum pepsinogen for that animal before infection.

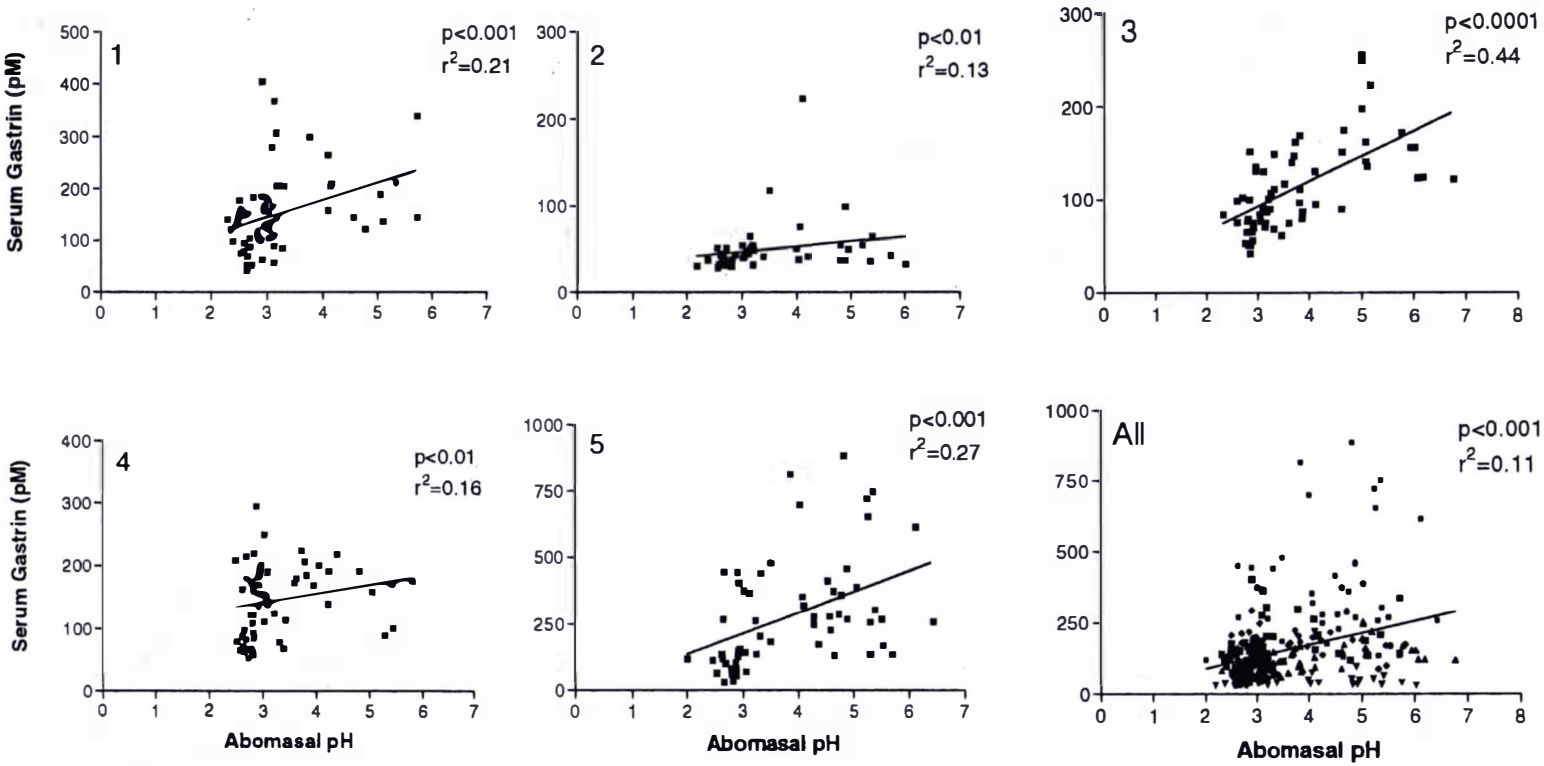

Figure 3.3. Correlation between serum gastrin and abomasal pH for Sheep 1-5 infected on Day 7 with 150 000 exsheathed L3 *O. circumcincta* and Day 18 with 100 000 sheathed L3 *O. circumcincta.* Serum gastrin is plotted against abomasal pH for each animal and for all data. P and r^2 values given are for Spearman correlation of the data. Data are from post infection samples only.

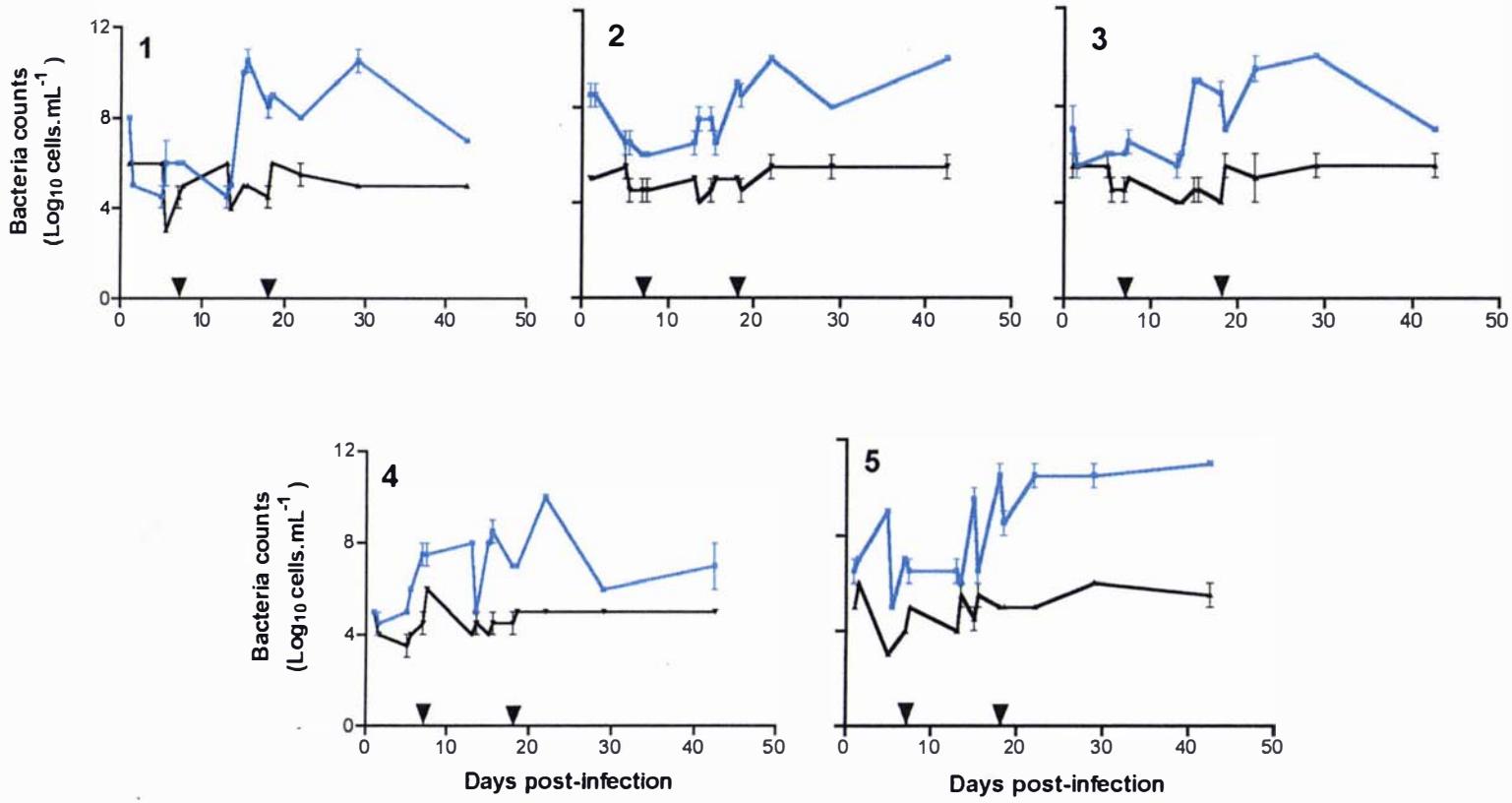

Figure 3.4. Aerotolerant (——) and anaerobic (—) bacterial counts for Sheep 1-5 infected on Day 7 with 150 000 exsheathed L3 *O. circumcincta* and Day 18 with 100 000 sheathed L3 *O. circumcincta.* Arrowheads indicate infection times. Error bars represent SEM for duplicate enumerations.

3.3 Results

3.3.1 Experiment 1, LPI-1

3.3.1.1 Abomasal pH, serum gastrin, and pepsinogen, abomasal peptic activity

Before infection, the group mean and upper limits of normal values were established as: abomasal pH2.73 and 3.10 respectively; serum gastrin concentration 64 and 112pM and serum pepsinogen concentration 0.63 and 2.01i.u. (n=55). For all three parameters, before and after feeding samples were not significantly different ($p>0.05$); after infection, there was marked variation in responses between animals both in magnitude and timing (Figures 3.1 and 3.2). In general, all three parameters began increasing around Day 15-17, with larger changes from Day 19 onward. Notable were the failure of serum gastrin to rise in Sheep 2 although abomasal pH increased substantially, and the low serum pepsinogen concentration both before and after infection in Sheep 4. After infection, feeding increased abomasal pH in Sheep 2 and 5, raised serum gastrin concentration in Sheep 2 and serum pepsinogen concentration in Sheep 5.

A period of raised abomasal pH without elevation of serum gastrin concentration was seen in all five animals, either around Day 17 (Sheep 1, 3 and 5) or Days 22-27 (Sheep 2, 3 and 4) (Figure 3.1). At other times after infection, however, serum gastrin remained elevated after abomasal pH had returned to pre-infection levels. These sources of divergence led to an overall poor correlation between abomasal pH and serum gastrin for individual animals (Figure 3.3).

Abomasal peptic activity was variable both before and after infection. There was no correlation between serum pepsinogen concentration and abomasal peptic activity.

3.3.1.2 Abomasal bacteria

The densities of viable aerotolerant bacteria in the abomasa of the five sheep, each sampled 15 times, varied from 10^3 to 10^6 cells.mL^{-1} (Figure 3.4).

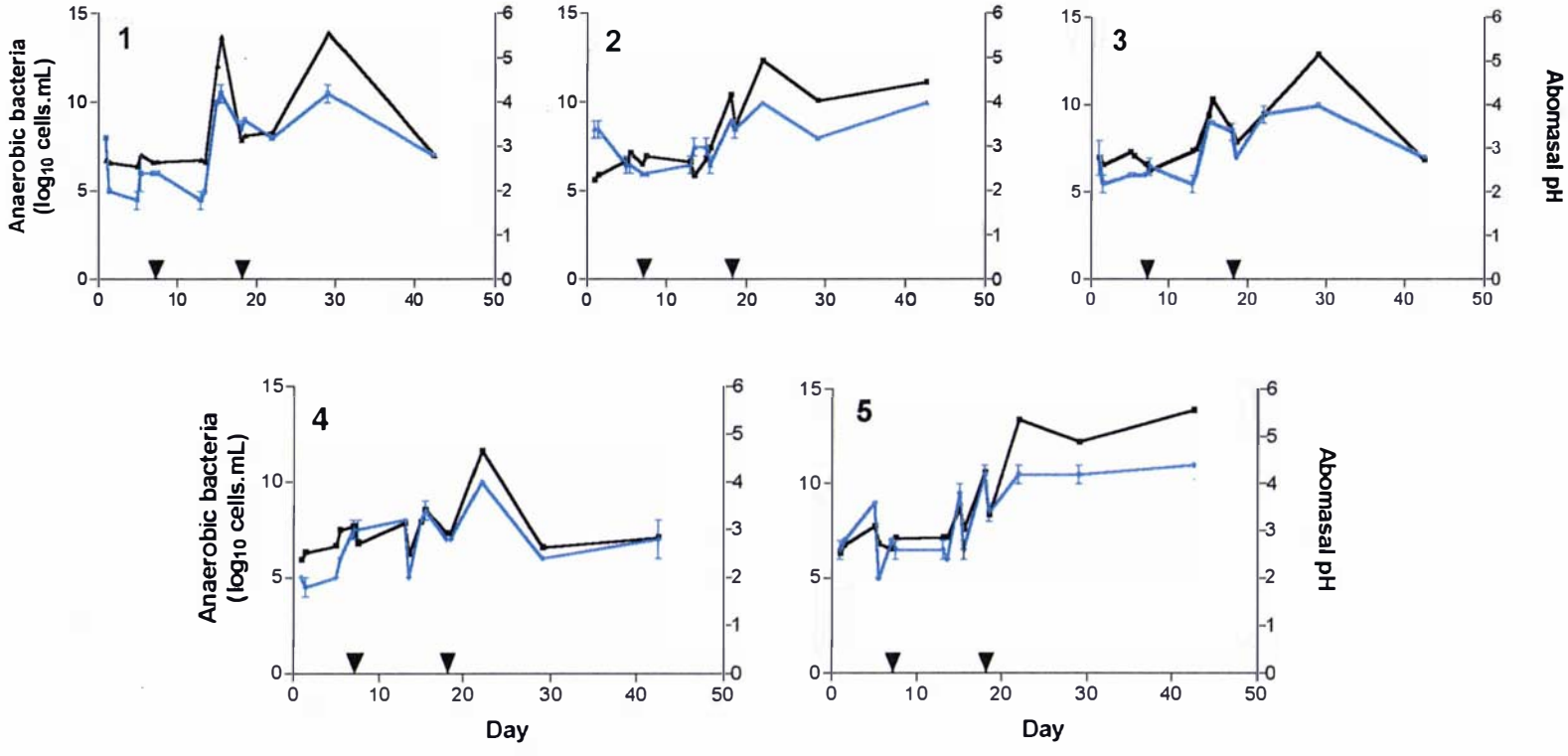

Figure 3.5. Anaerobic bacterial counts (—) and abomasal pH (—) for Sheep 1-5 infected on Day 7 with 150 000 exsheathed L3 *O. circumcincta* and Day 18 with 100 000 sheathed L3 *O. circumcincta*. Infection times are indicated with arrowheads. Error bars on enumeration data points are the SEM for that sample.

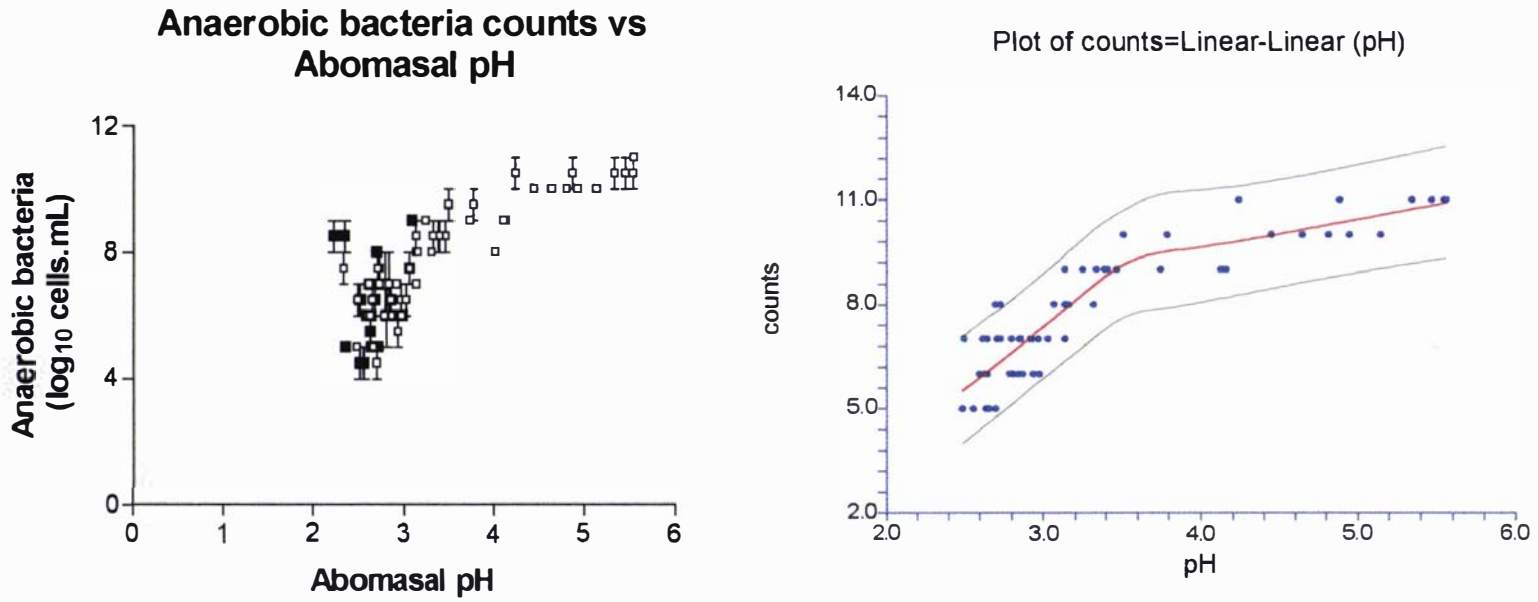

Figure 3.6. Relationship between abomasal pH and anaerobic bacterial counts using combined data from Sheep 1-5 infected on Day 7 with 150 000 exsheathed L3 *O. circumcincta* and Day 18 with 100 000 sheathed L3 *O. circumcincta.* Left. A plot of pre- (■) and post- (□) infection anaerobic bacterial counts versus the pH of the sample. All counts were estimated in duplicate, and where error bars are shown, they represent the SEM when duplicate results differed. Right. The linear-linear segmented model that best fitted these data is shown. The model contains two linear regression lines representing the two phases in the data which are joined, forming the curve shown. The two lines are given by the equations: anaerobic bacterial number = a1 + b1(pH) if pH<J; if pH>J, counts = a2 + b2(pH). J (join point between lines) = 3.53, a1 = -3.252, b1 = 3.521, a2 = 6.2, b2 = 0.846; r^2= 0.79, p<0.001. Upper and lower confidence intervals of the line are also shown.

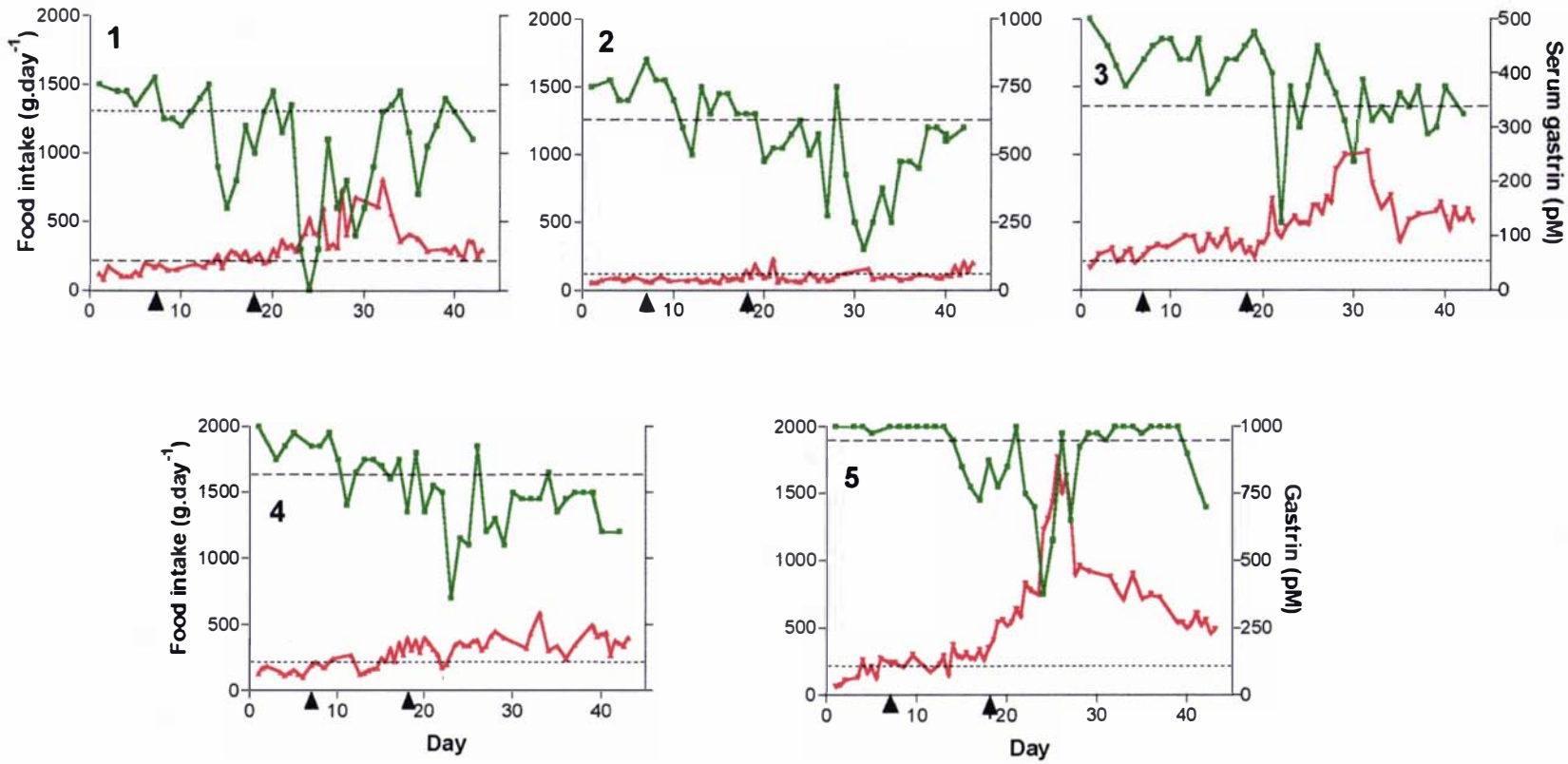

Figure 3.7. Daily food intake (—) and serum gastrin (—) in Sheep 1-5 infected on Day 7 with 150 000 exsheathed L3 *O. circumcincta* and Day 18 with 100 000 sheathed L3 *O. circumcincta*. Arrowheads indicate infection times. The upper horizontal dotted lines represent 2 SD below the mean values of food intake before infection, and the lower dotted lines 2 SD above the mean values for serum gastrin before infection.

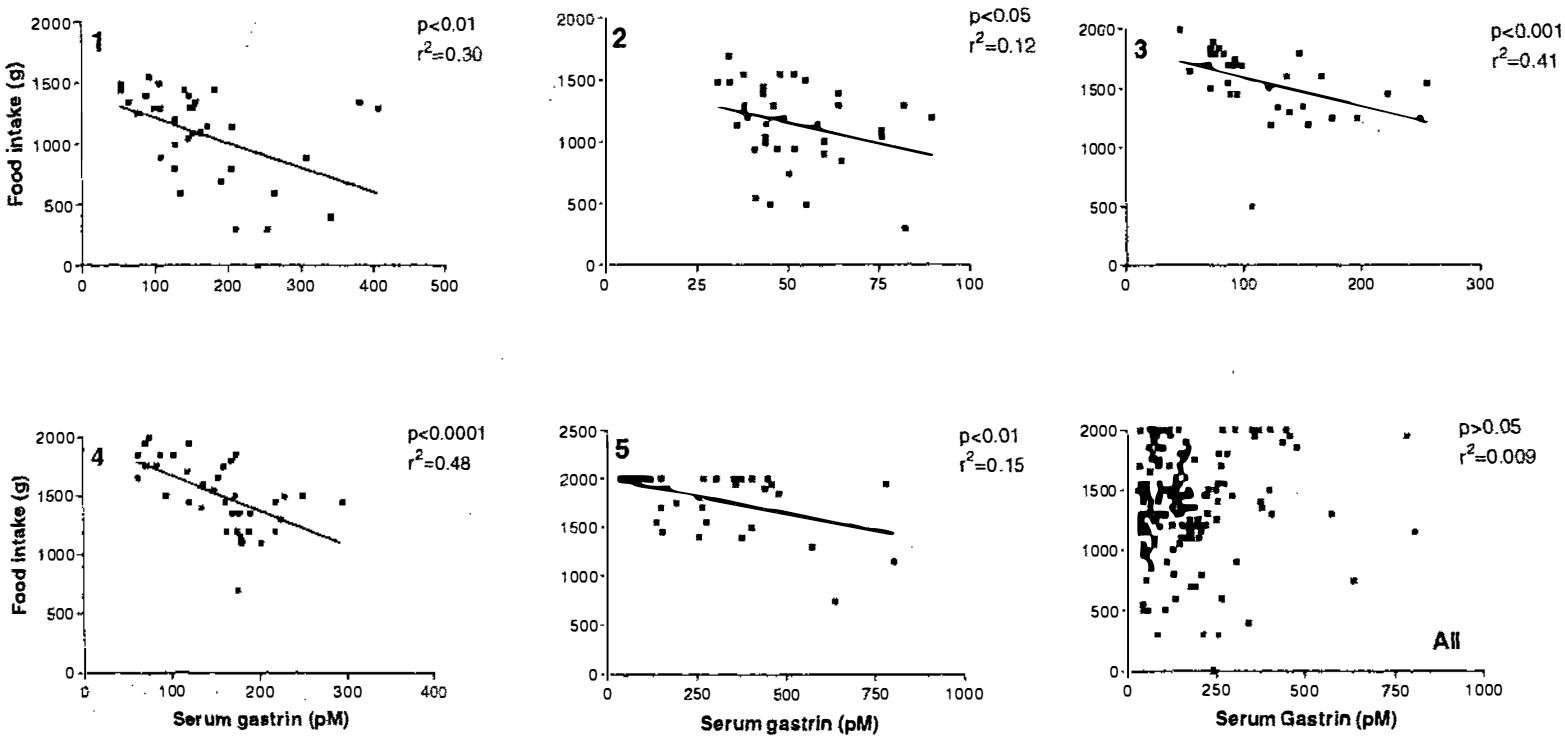

Figure 3.8. Correlation between serum gastrin and daily food intake for Sheep 1-5 infected on Day 7 with 150 000 exsheathed L3 *O. circumcincta* and Day 18 with 100 000 sheathed L3 *O. circumcincta.* Food intake is plotted against serum gastrin for each animal and for all data. The p and r^2 values given are for Spearman correlation of the data. Data are from post infection samples only.

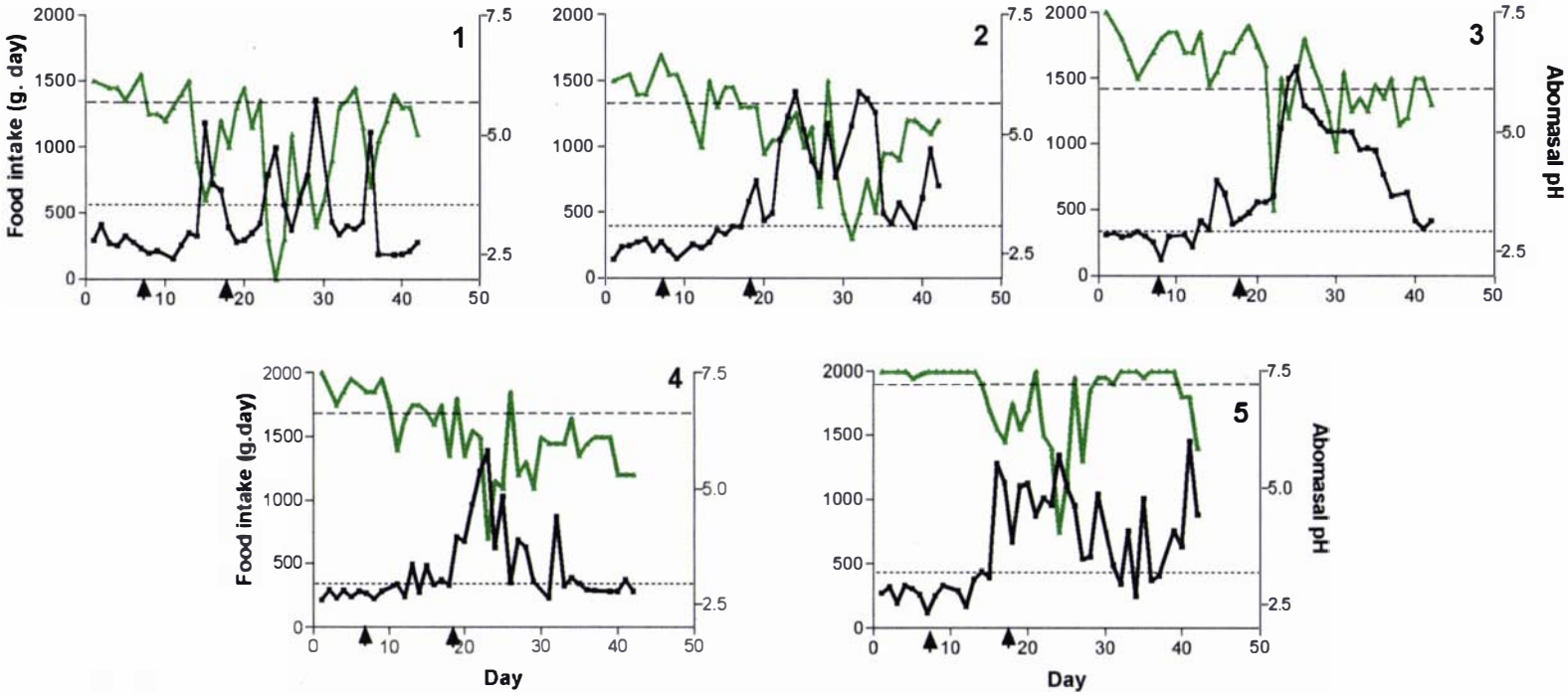

Figure 3.9. Daily food intake (—) and abomasal pH (—) in Sheep 1-5 infected on Day 7 with 150 000 exsheathed L3 *O. circumcincta* and Day 18 with 100 000 sheathed L3 *O. circumcincta*. Arrowheads indicate infection times. The upper horizontal dotted lines represent 2 SD below the mean values of food intake before infection, and the lower dotted lines 2 SD above the mean values for abomasal pH before infection.

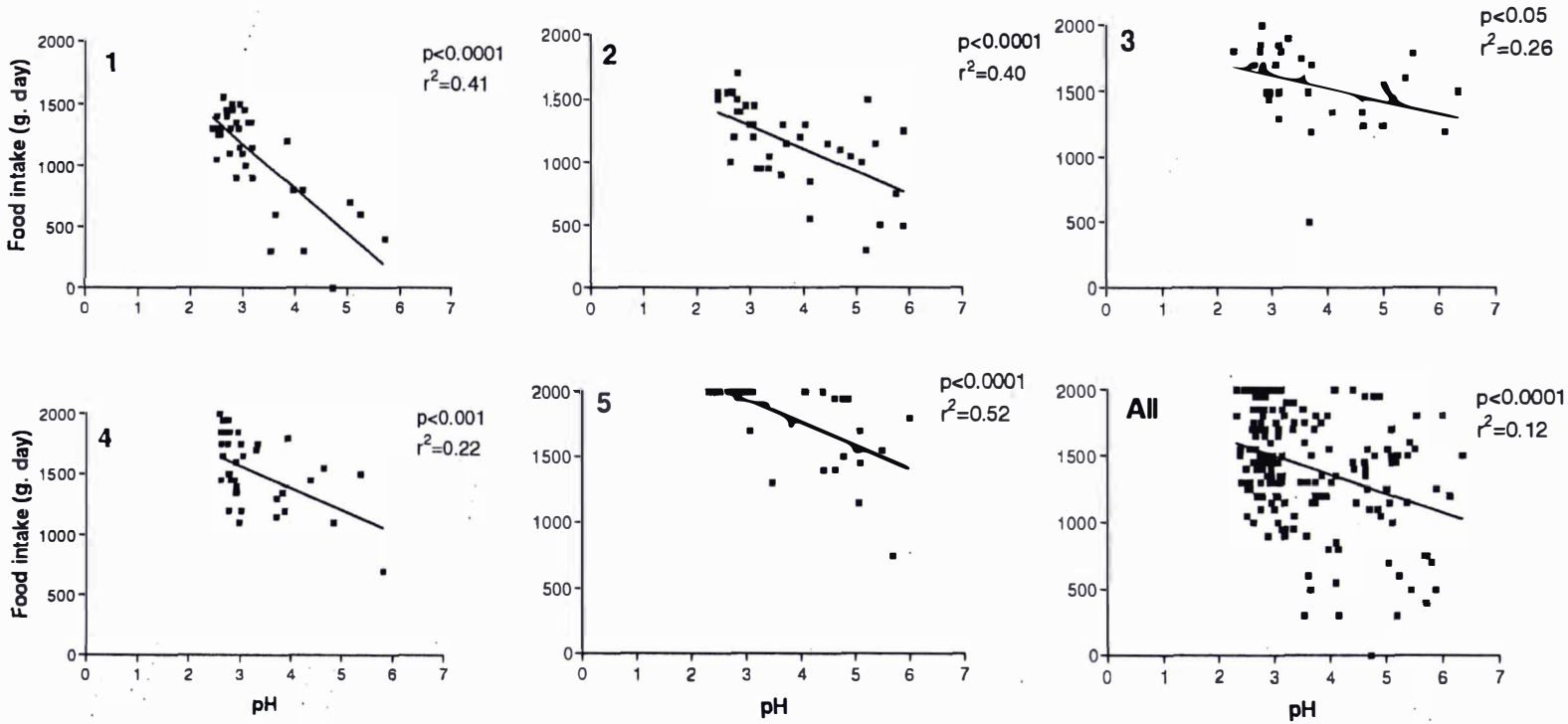

Figure 3.10. Correlation between abomasal pH and daily food intake for Sheep 1-5 infected on Day 7 with 150 000 exsheathed L3 *O. circumcincta* and Day 18 with 100 000 sheathed L3 *O. circumcincta.* Food intake is plotted against abomasal pH for each animal and for all data. The p and r^2 values given are for Spearman correlation of the data. Data are from post infection samples only.

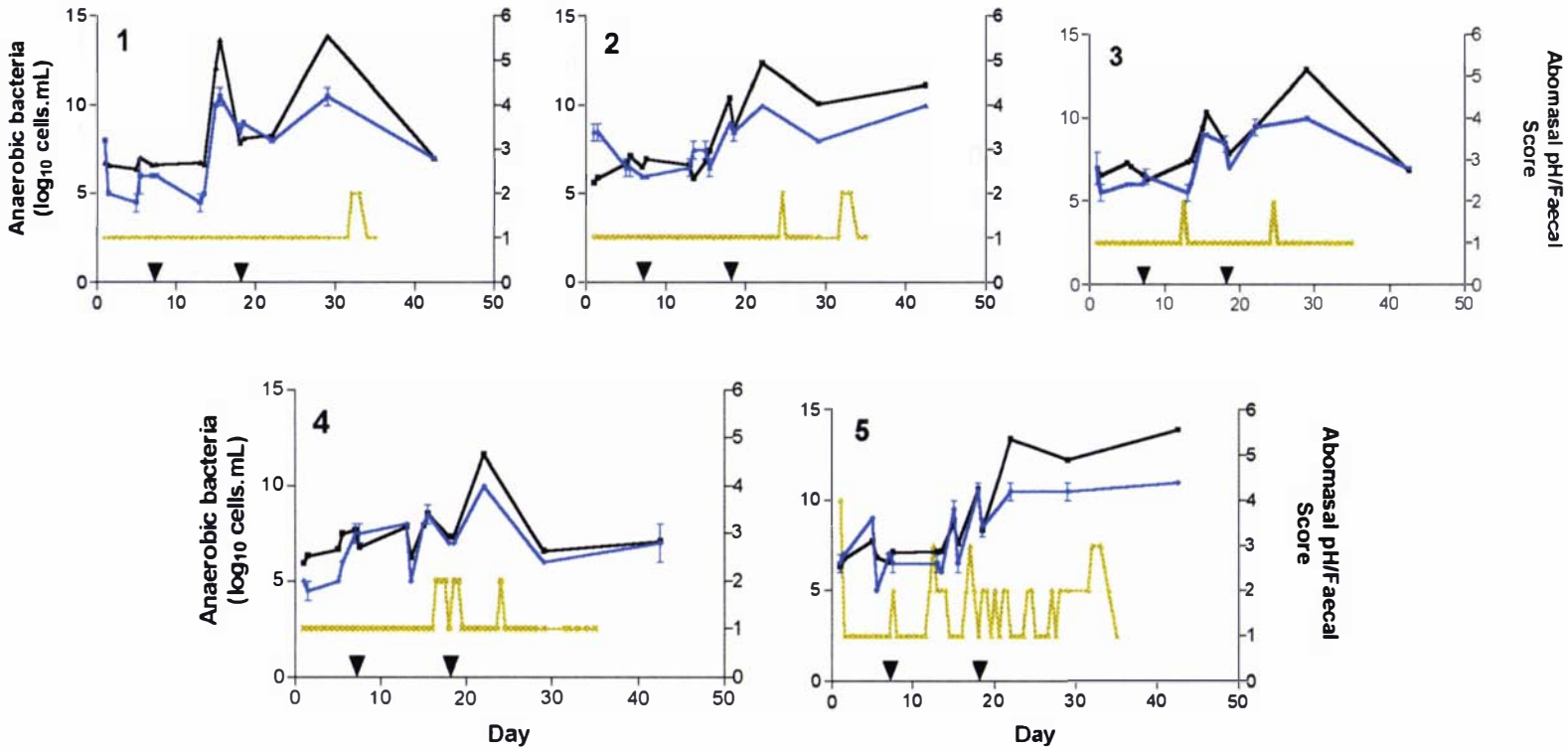

Figure 3.11. Anaerobic bacterial count (—), abomasal pH (—) and faecal consistency (—) for Sheep 1-5 infected on Day 7 with 150 000 exsheathed L3 *O. circumcincta* and Day 18 with 100 000 sheathed L3 *O. circumcincta.* Infection times are indicated with arrowheads. Error bars on enumeration data points are the SEM for duplicate estimates of the sample.

The aerotolerant bacterial population densities did not change significantly following parasitism. The numbers of viable anaerobic bacteria in the abomasa before infection ranged from 10^5 to10^9 cells.mL^{-1}, increasing post-infection in all sheep to maximum values of 10^{10} to 10^{11} cells.mL^{-1} (Figure 3.4). In each animal, raised abomasal pH was closely associated with increased anaerobic bacterial populations (Figure 3.5). The piecewise polynomial equation that best fitted the pooled data from pre- and post-infection samples was a segmented linear-linear model with a joining point at pH 3.52±0.18 (Figure 3.6). There was no correlation between anaerobic bacterial counts and abomasal peptic activity within animals or in the pooled data for all animals.

3.3.1.3 Food intake

The daily food intake varied between animals in the control period (Figure 3.7). After infection, food intake decreased markedly in four of the five sheep, for two separate periods in Sheep 1 and 5 and for a single 8-10 day period in Sheep 2 and 4 (Figure 3.7). There was no correlation between food intake and bacterial counts and a negative correlation between food intake and serum gastrin concentrations for individual animals (Figure 3.8). Food intake did not correlate more closely with abomasal pH than with serum gastrin (Figures 3.9 and 3.10).

3.3.1.4 Faecal consistency

Faecal consistency is shown in Figure 3.11. During the control period all animals were scored at 1 indicating faeces were pellets. In general, there was little change in this after infection: two animals (Sheep 4 and 5) produced soft pellets that adhered to each other. For Sheep 5, this was noted after the first infection with 150 000 exsheathed L3 and the second infection 100 000 sheathed L3 larvae, but for Sheep 4 soft faeces were only noted after the second infection. Sheep 5 occasionally produced faeces which scored at 3. Diarrhoea did not occur. There was no relationship between faecal consistency and bacterial counts, or between faecal consistency and abomasal pH. Softer pellets were observed during the period of greatest hypoacidity after the second infection in Sheep 1, 2 and 3, though pellet hardness was not scored.

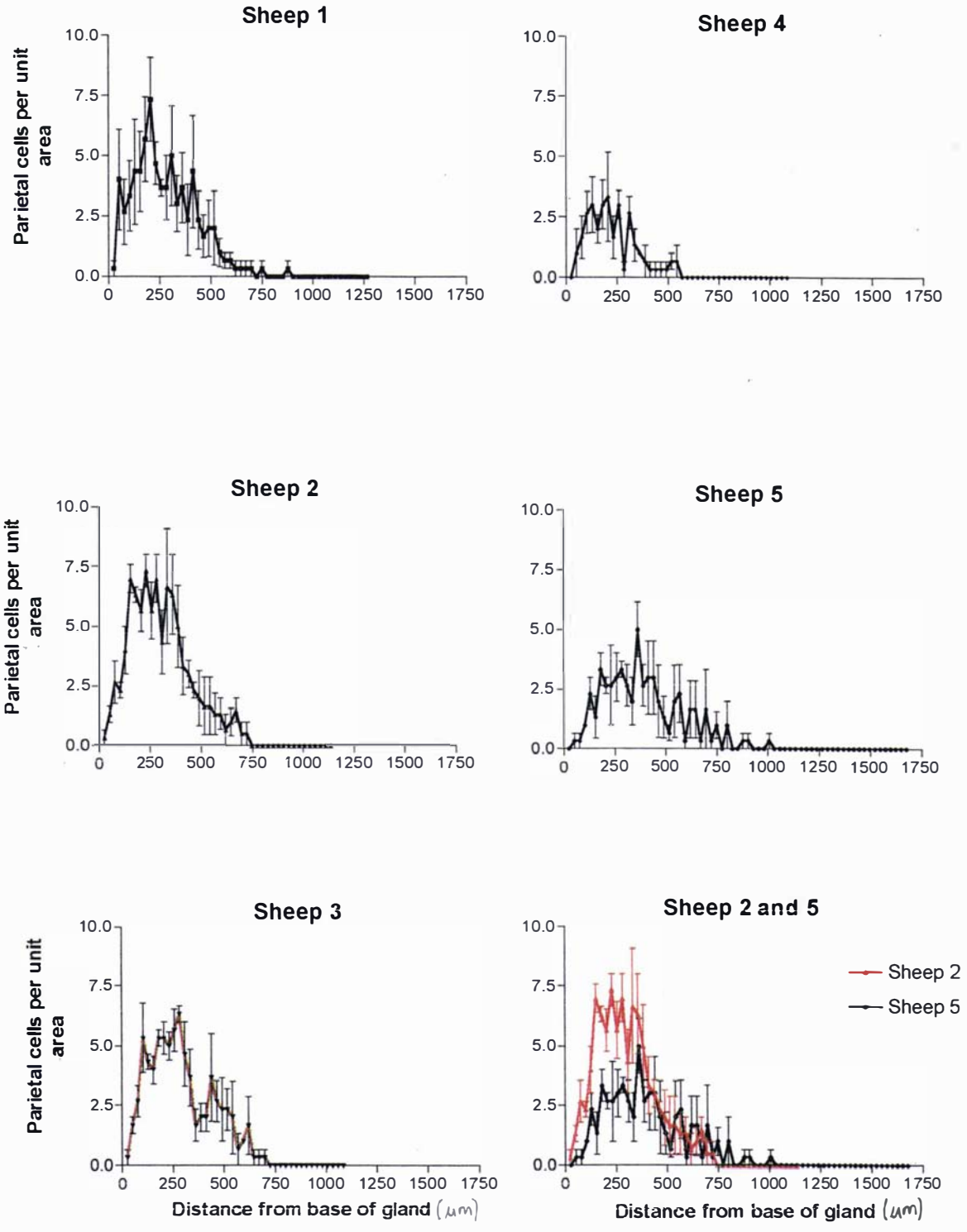

Figure 3.12. The number of parietal cells per unit area (mean±SEM) (25.8μm deep by 258μm wide) in columns of fundic mucosa for Sheep 1-5 infected on Day 7 with 150 000 exsheathed L3 *O. circumcincta* and Day 18 with 100 000 sheathed L3 *O. circumcincta*.

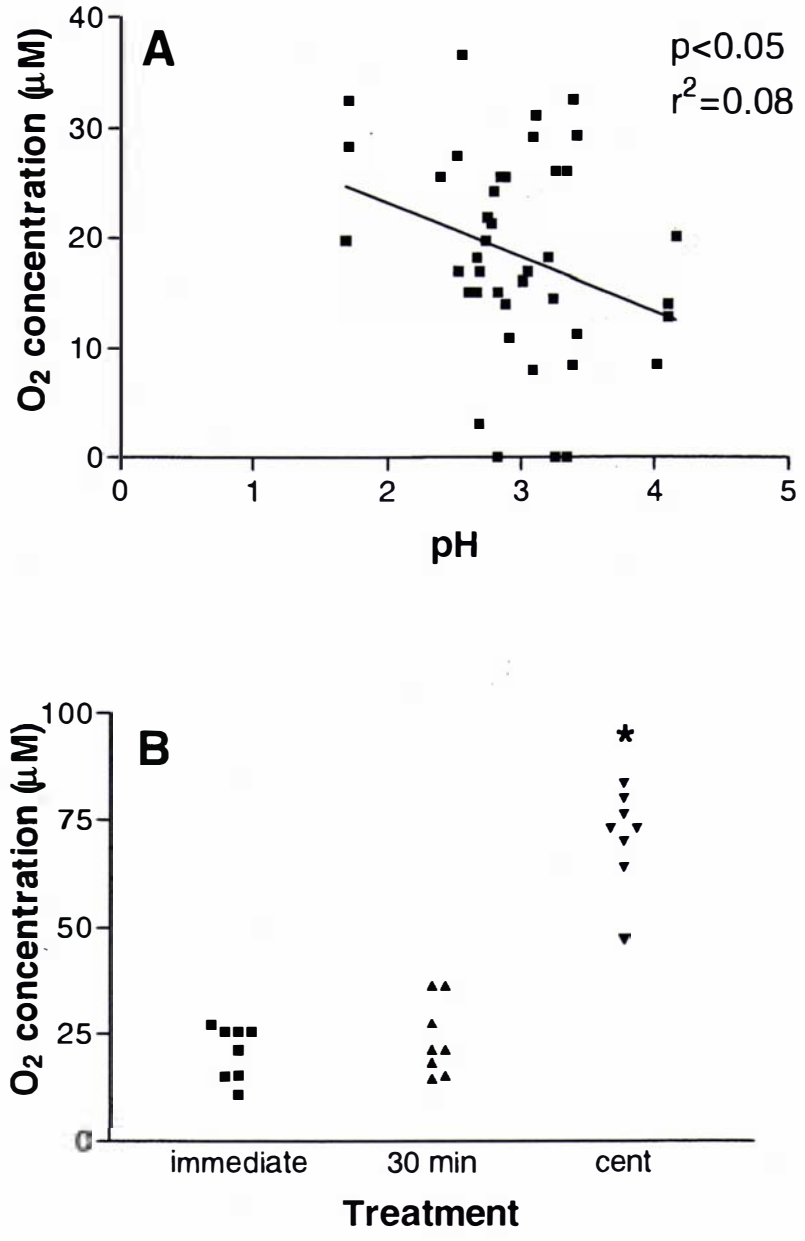

Figure 3.13. Oxygen concentration in abomasal contents from 8 sheep parasitised with L3 *O. circumcincta*. A: the correlation between oxygen concentration and abomasal pH in samples taken from 8 animals on 9 separate days. The p and r^2 values are for a Spearman one tailed correlation are shown. B: the oxygen concentration in the supernatant of abomasal samples centrifuged at 2000*g* for 30 minutes. *= $p<0.05$, centrifugation (cent) resulted in an increase in oxygen levels when compared with measurements made immediately after the sample was taken (immediate), and when the sample was left standing, sealed, for 30 minutes (30 min).

3.3.1.5 Parasitology

No eggs were detected in the faeces prior to infection or until 25 days after the first infection, when a few eggs were detected from Sheep 5. Seven days later, all sheep were excreting eggs. The maximum faecal egg count (FEC) recorded was 550 eggs per gram (e.p.g). Worm counts at necropsy were 12 350, 3 380, 2 180, 1 520 and 12 050 adult worms and 40, 140, 40, 90 and 0 immature stages in Sheep 1-5 respectively.

3.3.1.6 Gross pathology and histology

At necropsy, lesions were apparent in all animals in the pylorus, but there were few in the fundus. In Sheep 2, there was gross thickening of the mucosa, especially in the pylorus.

Numbers of G cells per field were: Sheep 1, 21.33±1.76; Sheep 2, 5.83±1.44; Sheep 3, 15.67±1.78; Sheep 4, 13.67±1.24; Sheep 5, 16.67±1.24. Sheep 2 had fewer G cells per field than all other animals ($p<0.05$), and Sheep 1 had more G cells per field than Sheep 4 ($p<0.05$). Otherwise, there were no differences between animals ($p>0.05$). Some eosinophils stained weakly for gastrin.

The distribution of parietal cells in fundic glands of each animal at necropsy is shown in Figure 3.12. There was no difference in the number of parietal cells per field between animals, but Sheep 5 had thicker fundic mucosa than Sheep 2 and Sheep 4.

3.3.2 Experiment 2 (LTI)

3.3.2.1 Oxygen concentration

Oxygen concentration in all abomasal samples was below 40μM, whether sample pH was normal or elevated. There was no significant difference in oxygen concentration between days ($p>0.05$) and only a poor correlation between abomasal pH and abomasal oxygen concentration (Figure 3.13A). ANOVA revealed that there was significant variation in oxygen concentration between only two animals ($p<0.05$). The mean oxygen

concentration for each animal varied from 11 to 36μM, though the range of values was similar for all.

The stability of oxygen concentration in oxygenated abomasal samples over time was no different from those observed for oxygenated water, or in the presence of sodium hypochlorite ($p>0.05$). Oxygen concentration did gradually decline over time from all saturated samples.

Centrifugation significantly increased oxygen concentration. Leaving abomasal contents standing in a sealed tube for 30 minutes did not affect oxygen concentration (Figure 3.13B).

3.4 Discussion

These experiments examined the relationships between the hypergastrinaemia of abomasal parasitism, as well as changes in the abomasal environment and depression of appetite. In particular, the abomasal bacterial densities, both aerotolerant and anaerobic, were compared with serum gastrin levels to test the hypothesis that microbial products could be involved in altering gastrin secretion. This was not clearly evident in this experiment, nor did hypergastrinaemia appear the principal cause of anorexia in these animals. The study, however, did reveal that an increase in the pH of abomasal contents, by as little as one pH unit, had a surprisingly marked effect on anaerobe survival. This may have nutritional implications for ruminants, which use microbial protein as an important source of dietary nitrogen.

3.4.1 Establishment and effects of parasitism

The purpose of the LPI-1 experiment was not specifically to follow the course of events in a single infection, but to examine concurrent changes in abomasal bacterial densities and serum gastrin as abomasal pH increased. Similarly, the second experiment was designed to determine whether there was a correlation between abomasal pH and oxygen concentration, rather than to follow changes during an infection. In the first experiment, parasitism was initially established by administering exsheathed larvae directly into the

abomasum, a protocol which had proved highly successful in a previous study (Lawton *et al.,* 1996). When the effects on abomasal pH were less than expected 10 days post infection (Day 17), a dose of sheathed larvae was given on Day 18. In all five sheep, changes in abomasal pH and serum gastrin and pepsinogen concentrations indicated establishment of parasitism. Prior to this experiment, two animals (Sheep 4 and 5) had been infected with *T. colubriformis,* then treated with anthelmintic. As this had no apparent effect on abomasal function, nor was there any evidence that these animals reacted to infection with *O. circumcincta* any differently from the parasite-naïve animals, their data are also included.

3.4.1.1 Abomasal microbial populations

Despite the low pH, even the unparasitised abomasum harboured substantial populations of bacteria (Figure 3.4), both aerotolerant and anaerobes. The range of population densities of aerotolerant bacteria in this study was from 10^3-10^6 viable cells.mL^{-1}, similar to that in abomasal samples from two *O. ostertagi*-infected calves ($7x10^4$ to $4x10^6$ cells.mL^{-1}) (Jennings *et al.,* 1966). Their research reported an increase in the density of aerotolerant bacteria when abomasal pH rose from 7.0 to 7.4 during the period of severe diarrhoea. In contrast, in the present study, the numbers of aerotolerant abomasal bacteria were not related to parasitism, nor to abomasal pH.

The population densities of anaerobic bacteria increased dramatically as abomasal pH increased. The densities reached levels around 10^{10} cells.mL^{-1}, levels similar to those in the rumen (Leedle and Hespell, 1980). These numbers are in agreement with observations in sheep infected with massive doses of *H. contortus* larvae in which anaerobic bacterial densities increased to 10^9 cells.mL^{-1} when the pH increased to 6 and over (Nicholls *et al.,* 1987). Of particular interest in the present study was the finding that most of the increase in abomasal anaerobic bacteria occurred over only a small pH range (2.5-3.5) (Figures 3.5 and 3.6). Further increases in pH beyond 3.5 resulted in relatively small increases in anaerobic bacterial numbers (Figures 3.5 and 3.6). The simplest explanation for the increased anaerobic bacterial population is increased survival of rumen anaerobes because of decreased lysis at higher abomasal pH. An alternative explanation is bacterial growth in the abomasum.

This is considered unlikely because growth to densities of 10^{10} cells.mL^{-1} in the abomasum would require a large supply of growth substrate and it is not clear where this would come from. In addition, the flow rate of digesta though the abomasum is high, giving little opportunity for bacterial populations to become established.

3.4.1.2 Abomasal oxygen concentration

Oxygen concentrations did not change during parasitism in contrast to results presented by Nicholls *et al.* (1987). Nicholls *et al.* (1987) reported that the abomasal oxygen tension decreased as the pH increased along with anaerobic microbial numbers in the abomasum during parasitism. They observed that the contents of the unparasitised abomasum were oxygenated to similar levels to those in venous blood, but became more anaerobic during infection, which may have allowed anaerobic bacteria to survive and proliferate. Results presented for the sheep in LPI-1 show aerotolerant bacterial numbers to be constant before and during infection (Figure 3.4). In the LTI experiment, there was little variation in oxygen tension, which remained low despite large fluctuations in abomasal pH (Figure 3.13A). Nicholls *et al.* (1987) collected their samples for anaerobic bacteria quantification using vacuum sealed tubes. For results presented here, samples were exposed to air very briefly. This is not expected to have influenced results however, as transfer of oxygen between liquid and air is not rapid and the samples were anaerobic. It would be expected that oxygen would diffuse into the abomasal solutions as the concentration of oxygen in the air is greater than that in the samples. The centrifugation of samples could be one possible explanation for the higher oxygen concentrations reported by Nicholls *et al.* (1987). Oxygen concentrations in the supernatants were greater than those of whole abomasal contents in this experiment (Figure 3.13B), though the cause of this is uncertain. The ability to reduce oxygen concentrations of abomasal fluid was tested with fluid of pH4, as this would be expected to have near maximum numbers of bacteria. As oxygenated abomasal contents did not reduce the oxygen concentration more than oxygenated water or abomasal contents with sodium hypochlorite, abomasal bacteria did not appear to have the ability to reduce oxygen levels of abomasal fluid. Therefore, elevated abomasal pH

alone appears to cause the increased bacterial numbers in the abomasum during parasitism in studies presented here.

3.4.1.3 Hypergastrinaemia

Previously, it was suggested that depression of serum gastrin during severe abomasal hypochlorhydria in parasitised sheep may be caused by increased microbial populations in the abomasum (Lawton *et al.,* 1996). In this study, there were several periods when abomasal pH was increased, but serum gastrin remained within normal limits (Figure 3.1) and anaerobic bacteria counts were high (Figure 3.5). However, there were other periods when anaerobic bacteria densities and pH were both high, but gastrin concentrations were also raised. This suggests that bacterial involvement is not essential to the lack by correlation of pH and gastrin seen in this study and previously (Lawton *et al.,* 1996). It is possible that the luminal interference in gastrin secretion proposed by Lawton *et al.* (1996) is due to an overgrowth of a population of bacteria more intimately associated with the mucosa than bacteria in the abomasal contents. Bacterial colonisation of the gastric pits has been reported in *post mortem* studies of calves subject to chronic diarrhoea and illthrift, though interestingly these bacteria were not present in the pyloric glands or pits (Gunning, 2000). If a similar bacterial colonisation occurred in the pyloric glands in the study of Lawton *et al.* (1996), these bacteria may have been more able to affect gastrin secretion than bacteria in the abomasal contents due to their closer proximity to the G cell.

Hypergastrinaemia has been proposed as a diagnostic marker for abomasal parasitism, either replacing, or in conjunction with, serum pepsinogen estimation (Fox *et al.,* 1988; Hilderson *et al.,* 1992). In the present study, the extent of the increase in serum pepsinogen concentration varied markedly in individual sheep (Figure 3.2) and was negligible in one animal (Sheep 4). This is in agreement with some previous studies on serum pepsinogen levels during abomasal parasitism (Lawton *et al.,* 1996; Scott *et al.,* 1998a). Similarly, serum gastrin levels were not elevated in all animals (Sheep 2) or were not elevated during some periods of the infection despite elevated abomasal pH. Therefore, serum gastrin appears not be a consistent indicator of abomasal parasitism.

Results at necropsy suggested that the site of larval development was the antrum rather than the fundus. While the fundus is generally observed to be the site of the histotrophic phase of development, predominantly antral infections have been previously reported (Sommerville *et al.,* 1954; Durham and Elliot, 1975). In addition, Armour *et al.* (1966) described infections where mucosal hyperplasia moved from being predominantly in the fundus to the antrum later in infection. Unfortunately, none of these studies examined serum gastrin concentration.

In LPI-1, Sheep 2 was the most extreme example in this study of an animal in which serum gastrin barely increased with parasitism. In this animal, serum gastrin was remarkably consistent and mostly within normal limits during parasitism, despite marked increases in pH. There was massive thickening of the antral mucosa at necropsy and histologically severe inflammation, which may have damaged the gastrin-secreting G cells, resulting in the lower serum gastrin concentrations. Reduced numbers of G cells were observed in this animal compared with other animals in that study at necropsy. Conversely, more commonly a more moderate inflammation may play a significant role in the elevation of serum gastrin, as levels are higher than would be predicted due to increased pH alone. In parasitised sheep, peak serum gastrin concentrations of over 300pM, as seen in this experiment, are not unusual (Fox *et al.,* 1988; Nicholls *et al.,* 1988; Lawton *et al.,* 1996; Simpson *et al.,* 1997; Simpson *et al.,* 1999) and even greater levels (2-3 fold) occurred in some animals (Sheep 3 and 5). In healthy, non-parasitised sheep, when abomasal pH was elevated by sodium bicarbonate infusion, even to pH5, serum gastrin did not exceed 100pM (Reynolds *et al.,* 1991). Mediators of inflammation such as histamine (Bado *et al.,* 1994; Schubert and Makhlouf, 1996), TNFα (Lehmann *et al.,* 1996; Weigert *et al.,* 1996) and IL1 (Weigert *et al.,* 1996) have been shown to stimulate gastrin release. The high levels of gastrin observed during parasitism may be due to the superimposition of inflammation on the absence of the acid-gastrin feedback loop and the fact that parietal cells are likely to be unresponsive.

Eosinophilia is a feature of nematode parasite infections. It has recently been reported that eosinophils bind gastrin (Nichols *et al.,* 1998; Praissman *et*

al., 1998). This binding was localised to the granular portion of the eosinophil cytoplasm (Nichols *et al.,* 1998; Praissman *et al.,* 1998) and was not due to CCK_B receptor binding (Nichols *et al.,* 1998), but rather binds a 15kDa protein which may be major basic protein (Praissman *et al.,* 1998). It has been suggested that this binding to a secretory protein may indicate that eosinophils play a role in neutralising, destroying or controlling gastrin (Nichols *et al.,* 1998) and attenuate gastrin levels regulating gastric acid secretion and mucosal growth (Praissman *et al.,* 1998).

Examination of fundic tissue samples collected at necropsy showed no evidence that hypergastrinaemia aided the recovery of parietal cells in this study, as proposed by Scott *et al.* (1998a, b). Comparing the parietal cell numbers and distribution to those reported previously (Scott *et al.,* 1998b, 2000), there is an obvious decrease in cell numbers per gland with infection, and the distribution resembles that observed after an infection with adult *O. circumcincta*. There is no evidence of greater proliferation of new parietal cells in any one animal. Recent studies in transgenic mice, where either the gastrin gene or the CCK_B receptor gene has been deleted, show that gastrin is necessary for acid secretion and parietal cell differentiation (Koh *et al.,* 1997). However, in the LPI-1 study, the distribution of parietal cells in fundic samples taken at necropsy was no different between animals, despite the fact one animal (Sheep 5) showed marked hypergastrinaemia, while another (Sheep 2) showed no hypergastrinaemia during infection. These results do not rule out the role of gastrin in parietal cell proliferation reported in transgenic mice (Koh *et al.,* 1997), but rather suggest that serum gastrin levels within the normal range may be sufficient to stimulate differentiation. Hypergastrinaemia may upregulate the activity of the remaining parietal cells during the early stages of recovery from infection, enabling greater acid output, though there is no direct evidence of such activity in this study.

It was also noticeable that there was a marked proliferation in the fundic pits, in which parietal cells were absent. This was particularly clear in Sheep 5, the animal with marked hypergastrinaemia, which had thicker fundic mucosa than Sheep 2, which exhibited no hypergastrinaemia and Sheep 4, which exhibited only mild hypergastrinaemia. Fox (1997) reported that omeprazole

treatment in cattle resulted in similar mucosal growth responses to those observed in parasitised animals. In transgenic mice, hypergastrinaemia has been shown to coincide with increased fundic mucosal thickness (Wang *et al.,* 1996; Konda *et al.,* 1999), but is not essential, as fundic proliferation is unaffected by gastrin gene knockout (Koh *et al.,* 1997). However, Konda *et al.* (1999) reported that hypergastrinaemia was associated with a hypertrophic fundus, with elongated pits but normal sized fundic glands with normal, not increased, parietal cell numbers. The results of Konda *et al.* (1999) are at variance with the more commonly accepted model, that gastrin increases parietal cell differentiation rather than proliferation, possibly due to greater increases in blood gastrin concentration or the presence of gastrin producing cells in the fundus. However, the morphology reported by Konda *et al.* (1999) is not dissimilar from that observed in Sheep 5. Therefore, marked hypergastrinaemia may cause increased pit proliferation suggesting that gastrin may be partly responsible for mucosal growth during parasitism.

All observations concerning pathology need to be interpreted with a degree of caution, as the LPI-1 study only involved five animals, with one sample only taken from each region of each animal.

3.4.1.4 Food intake and nutrition

Anorexia commonly occurs during gastrointestinal parasitism of sheep (McLeay *et al.,* 1973; Anderson *et al.,* 1976a) and cattle (Fox *et al.,* 1989a, b) and has been attributed to hypergastrinaemia. In this study, inappetance did not correlate well with any of the parameters measured. Serum gastrin levels correlated poorly with food intake (Figure 3.8), unlike the close association observed in parasitised calves (Fox *et al.,* 1989a). One sheep had a reduced appetite without hypergastrinaemia. It appears that although serum gastrin may play some role in controlling food intake, other factors such as the inflammatory mediator TNFα (Holden and Pakula, 1996) or gastric pain may be important.

It is noticeable that the reduction in food intake appears to occur mainly when the abomasal pH first becomes elevated and generally remained depressed for only a few days (Figure 3.9). As serum gastrin did not correlate well with abomasal pH (Figure 3.3) and as pH is probably the best marker for

disruption of abomasal function, a correlation between abomasal pH and food intake was tested for each animal. These correlations for food intake and abomasal pH were no closer than serum gastrin concentrations and food intake for individual animals or pooled data. Better correlations for pH and food intake than food intake and gastrin were observed with animals 1, 2 and 5, but the opposite was true for animals 3 and 4 (Figures 3.8 and 3.10). Depression of food intake appeared to be a relatively short-term effect of infection, suggesting a single infection causes only short-term inappetance. Other studies have revealed significantly decreased food intake in infected animals on pasture, and this together with poor digestion may cause the reduction in liveweight gain observed as a result of parasitism (reviewed by Holmes, 1993; Coop and Kyriazakis, 1999).

The nature of the diet may have influenced the food intake results in this study. For example, the animals in this experiment were fed a diet well above the minimum recommended intake, which may have made the animals more resilient to infection. Further, in general observations and in three specific half hour observations, rumination was not observed in any of the animals, though eructation did occur. This was probably due to the nature of the lucerne pellet diet, which easily breaks down into small particles in water. It may well be that these animals were bored by their diet and consequently ate more than they would have had their diet contained more roughage. The dry nature of the lucerne chaff diet may also bias the faecal consistency scores (Figure 3.12), as animals fed on lucerne chaff have noticeably less fluid in the rumen than pasture fed animals.

Increased abomasal pH during parasitism is thought to have negative effects on protein digestion (Coop and Angus, 1981). Rumen microbes contribute over 50% of the total protein reaching the intestines of ruminants (reviewed by Broudiscou and Jouany, 1995), but how increased survival of bacteria in the abomasum affects utilisation of this protein is unknown. It seems likely that bacterial membranes must be lysed in order for intestinal enzymes to gain access to the majority of bacterial proteins, and that, in the unparasitised ruminant, most of the bacterial lysis occurs in the abomasum. It seems likely that decreased bacterial lysis due to abomasal hypoacidity would reduce the availability of bacterial protein to the host. This may become more

detrimental to the host during a mixed abomasal and intestinal nematode infection, where digestive efficiency of both gastrointestinal compartments is reduced. Combined with depression of appetite during *O. circumcincta* infection, reduced availability of nutrients may compromise the growth of the host even when diarrhoea is not prominent.

3.4.2 Summary

The number of anaerobic bacteria surviving in the abomasum of sheep parasitised with *O. circumcincta* increased rapidly with only a small elevation of abomasal pH, though aerobic bacterial numbers were unaffected. Experiments presented here showed anaerobic bacterial numbers were approximately 10^6 cells.mL^{-1} when abomasal pH is normal, between pH2.4 and 2.8, but rapidly increase to levels equivalent to those observed in the rumen, of 10^{-9} to 10^{11} cells.mL^{-1}, when abomasal pH increased to 3.5 and over. Survival of anaerobic bacteria related to elevation of abomasal pH, but not abomasal oxygen levels, which were low throughout infection, irrespective of the pH of the abomasal contents.

Survival of large numbers of bacteria does not affect serum gastrin levels, which are often elevated when anaerobic bacterial survival is maximal. Maximal survival of anaerobic bacteria occurs at much lower pH than pH5.5 at which Lawton *et al.* (1996) observed what was thought to be luminal interference with gastrin secretion. Thus, it is hypothesised that luminal interference with gastrin secretion may be caused by a chemical product rather than bacteria *per* se. Serum gastrin levels correlated poorly with abomasal pH (r^2 between 0.13 and 0.44 for individual sheep), with the onset of hypergastrinaemia lagging behind hypoacidity in most animals. This may have been due to tissue damage by the worms, as one animal in which hypergastrinaemia was never observed exhibited marked tissue damage in the antrum. Serum gastrin concentrations did not correlate well with food intake during infection (r^2 between 0.12 and 0.41 for individual sheep). Hypergastrinaemia did not appear to aid recovery of parietal cells, which were similar in number and distribution in all animals, regardless of the degree of hypergastrinaemia.

CHAPTER 4

GASTRIN INHIBITORY ACTVITY IN RUMEN AND ABOMASAL CONTENTS OF SHEEP

4.1 Introduction

The hypergastrinaemia typically seen during *O. circumcincta* infection in sheep (Anderson *et al.,* 1976a, 1981, 1985), during *O. ostertagi* infection in calves (Fox *et al.,* 1993), and *H. contortus* infection in sheep (Nicholls *et al.,* 1988; Simpson *et al.,* 1997) has been attributed largely to removal of acid inhibition of gastrin release (Fox *et al.,* 1993). Other factors appear to be involved, since in some animals, serum gastrin falls abruptly when the abomasal pH reaches or exceeds pH5.5, while in other animals serum gastrin remains elevated after abomasal pH returns to normal levels (Lawton *et al.,* 1996). In other animals, changes in serum gastrin levels do not parallel those in abomasal pH, especially where the parasites develop in the antrum (Chapter 3). These other factors may involve inflammatory mediators, depletion of tissue gastrin stores, worm products and chemicals produced by the gut flora.

Hypergastrinaemia has been shown to occur in calves infected with *O. ostertagi* (Purewal *et al.,* 1997) and sheep infected with *O. circumcincta* (Scott *et al.,* 1998a). Previous studies have shown that *O. circumcincta* E/S products are able to stimulate pepsinogen secretion (McKellar *et al.,* 1990b; Scott and McKellar, 1998) and inhibit acid secretion (Scott, Merkelbach and Simpson, unpublished), but have failed to show any effect on gastrin secretion *in vitro* (Haag, 1995; Lawton, 1995). In these latter two studies, however, contaminating abomasal microbes were believed to produce gastrin inhibition *in vitro.* Based on this observation, Lawton *et al.* (1996) suggested that luminal chemicals, possibly of microbial origin, might be responsible for the reversal in hypergastrinaemia at high abomasal pH.

In Chapter 3, abomasal bacterial numbers were shown to increase significantly with only small increases in abomasal pH, but their increased numbers *per se* did not appear to affect gastrin secretion. These findings did not, however, preclude the existence of a chemical inhibitor which is more sensitive to abomasal pH than are bacteria.

In this chapter, experiments testing the effects of abomasal contents collected from sheep infected with *O. circumcincta* on *in vitro* gastrin secretion are described. The aim of these experiments was to determine whether or not the abomasal contents contained an inhibitor of gastrin release and to examine the correlation between the presence of such an inhibitor and the fluctuation in serum gastrin and abomasal pH levels in the intact parasitised sheep.

4.2 Preliminary experiment: assessment of *in vitro* assay conditions

First, an assessment was made of the tolerance of tissues secreting gastrin in the *in vitro* gastrin system (described in Section 2.2.1) to variations in the pH and osmolarity of the basal medium. Next, samples of rumen fluid and abomasal contents from parasite-free animals and sheep from pasture were added to basal medium to assess responses and determine suitable concentrations for testing abomasal fluids *in vitro.*

4.2.1 Methods

4.2.1.1 Gastrin release: medium pH and osmolarity

The effect of varying the pH of the basal medium between pH7.20 and 7.60 on *in vitro* gastrin release was tested in four experiments. Control (A) solutions were maintained at pH7.40 and the pH of treatment (B) solutions was adjusted with 1M HCl or 1M NaOH.

In a further two separate experiments, the osmolarity of B solutions was varied between 260 and 380mOsm.L^{-1} by adding NaCl or distilled water to the

incubation medium. The osmolarities of the control (A) solutions were 320 and 330mOsm.L^{-1} in the two experiments.

4.2.1.2 Gastrin release: abomasal and rumen contents

Abomasal (n=3) and/or rumen (n=4) contents were collected from sheep which had been dosed with Ivermectin (Ivomec, Merial New Zealand Ltd), 0.7 mg.kg^{-1}, and brought indoors seven days before sampling. These sheep were fed lucerne chaff *ad libitum* and had water freely available. Two of the animals from which abomasal and rumen contents were taken were also the source of tissue for the *in vitro* preparation. Rumen contents (n=2) and abomasal contents (n=1) were also obtained from two pasture-fed sheep.

Abomasal and rumen contents were strained through a double layer of cheesecloth to remove large particulate matter. The resulting abomasal or rumen "liquor" was added to the basal medium to comprise 1, 2, 5, 10 or 20% of the final solution used for tissue incubation. All solutions were adjusted to pH 7.40±0.02 and an osmolarity of 320-340mOsm.L^{-1}.

4.2.1.3 Gastrin release: tissue viability

The effects of rumen contents (n=3) and abomasal contents (n=2) on gastrin secretion during subsequent incubation periods were tested, by incubating the tissues for a further two 30 minute incubation periods (called C and D solutions respectively), after the tissue had been exposed to rumen or abomasal fluids for 30 minutes. The ratios of C solution to A solution and D solution to A solution for the tissues previously incubated in gut fluid were then compared to the ratios of C/A and D/A solutions for control tissues.

4.2.1.4 Gastrin concentration in abomasal contents from unparasitised sheep

The gastrin content of five abomasal fluid samples were also tested. The samples were between pH2.4 and 2.8 and were obtained from five non-parasitised, lucerne fed sheep.

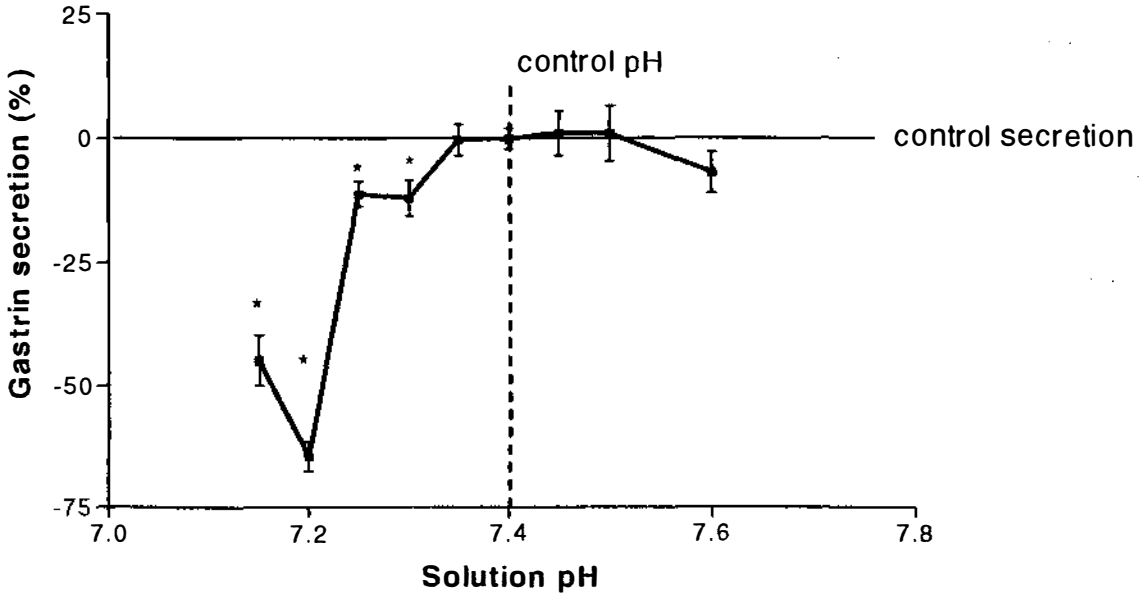

Figure 4.1. Effect of pH of the basal medium on *in vitro* gastrin secretion. Data are expressed as mean±SEM. The vertical dotted line indicating normal pH of the medium, *= p<0.05, treatment is different from control.

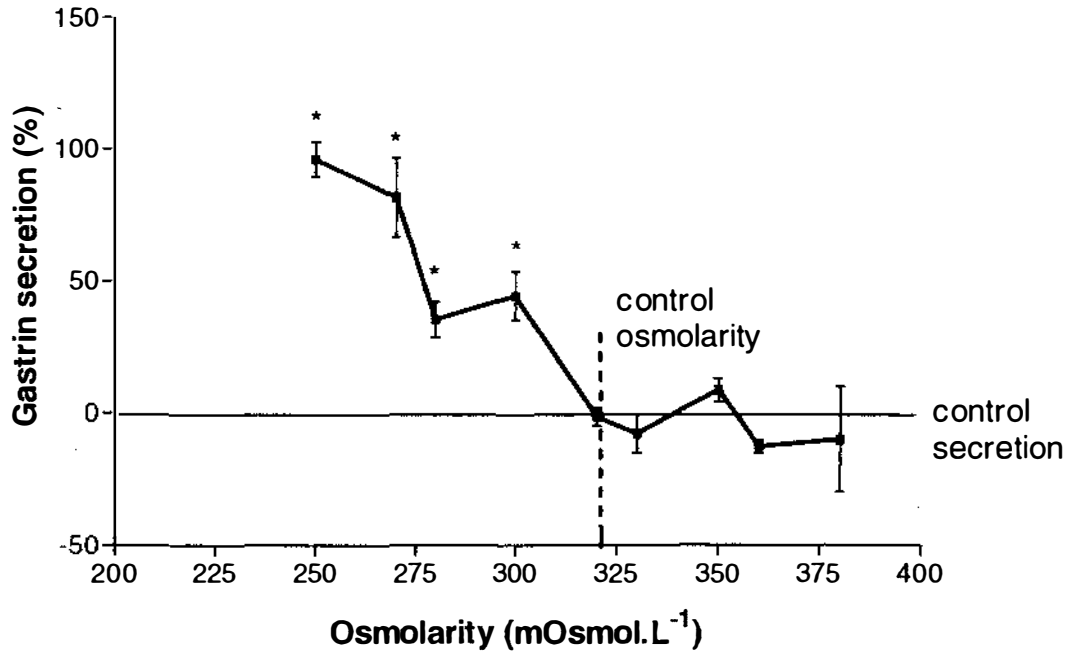

Figure 4.2. Effect of osmolarity of the basal medium on *in vitro* gastrin secretion. Data are expressed as mean±SEM. The vertical dotted line is the osmolarity of the standard solution, *=p<0.05, treatment is different from control.

4.2.1.5 Effect of rumen/abomasal contents on gastrin and somatostatin Radioimmunoassay (RIA)

The effect of a final concentration of 1% abomasal or rumen contents on maximum radioligand binding (zero) and non-specific binding (blank) in the gastrin and somatostatin radioimmunoassays (Appendix 1 and 2 respectively) solutions was tested.

4.2.1.6 Statistics

The effects of changed osmolarity of the medium, altered pH of medium, abomasal and rumen fluids on gastrin secretion were all analysed using UNIANOVA in SPSS version 9.0. Details of analyses and data considerations for UNIAVOA analysis are described Section 4.3.2.6.

4.2.2 Results

4.2.2.1 pH and osmolarity of the medium

Basal gastrin secretion was reduced when the pH of the medium was 7.3 or lower. Increasing the pH of the basal medium up to 7.6 had no significant effect (Figure 4.1).

Decreasing the osmolarity to 300mOsm.L^{-1} or lower, increased basal gastrin secretion, whereas increasing the osmolarity to 380mOsm.L^{-1} had no significant effect (Figure 4.2).

4.2.2.2 Gastrin concentration in abomasal contents

The gastrin content in all of the five abomasal samples from five non-parasitised sheep was less than 10pM. This compares to a gastrin concentration of between 100pM and 300pM in B solutions containing abomasal contents incubated with antral tissue.

4.2.2.3 *In vitro* gastrin response to rumen and abomasal contents

Abomasal contents from parasite-free animals fed lucerne chaff had a variable effect on the gastrin secretion, with responses between 6±4 to 101±25% (mean±SEM), but these increases were not significantly different

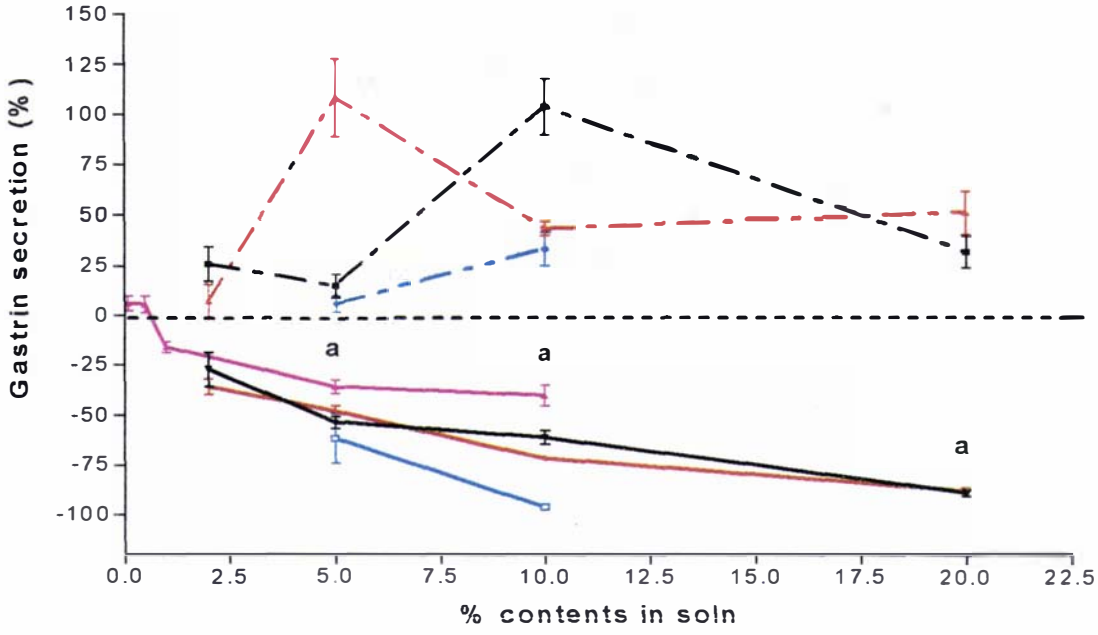

Figure 4.3. Effects of 0.1-20% abomasal or rumen contents from lucerne chaff-fed animals on *in vitro* gastrin secretion. Data are expressed as mean±SEM. Solid lines represent rumen contents, dashed lines abomasal contents in replicate experiments, a= secretion significantly less than control of all rumen samples at this concentration.

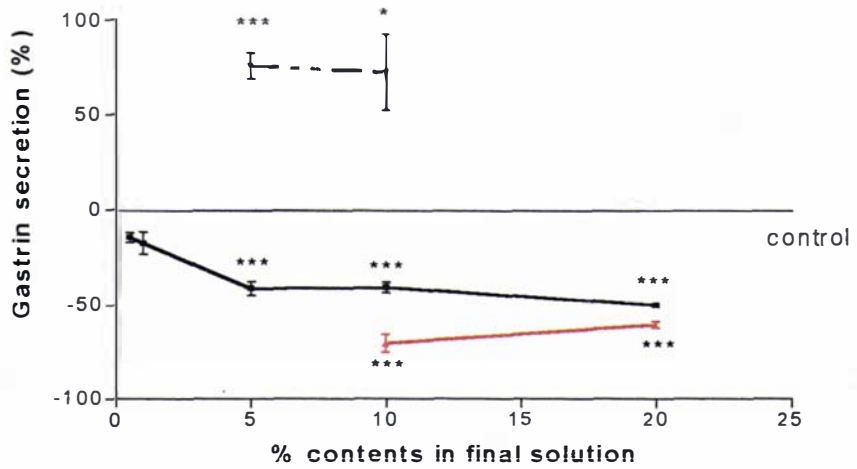

Figure 4.4. Effects of 0.5 to 20% abomasal or rumen contents from pasture-fed animals on *in vitro* gastrin secretion. Data are expressed as mean±SEM. Solid lines represent rumen contents, dashed lines abomasal contents in replicate experiments, *=p<0.05, ***p<0.001, significantly different from control secretion.

from control (Figure 4.3). The one abomasal sample from a pasture-fed animal stimulated ($p<0.05$) gastrin secretion (Figure 4.4). The pH of all abomasal contents was between 2.4 and 2.8.

Rumen contents were consistently inhibitory to gastrin secretion in concentrations of 5% and above (Figures 4.3 and 4.4). As the effect was near maximal at 10%, this concentration was used in the subsequent experiment.

In general, gastrin secretion was not affected by prior incubation with abomasal or rumen fluid. The ratio of C/A was greater for tissues incubated previously with solutions containing 20% rumen fluid than tissue incubated in control medium. This was because the C solution of tissues previously incubated in 20% rumen solution, which had higher gastrin concentrations than controls. Incubation of tissue in 10% rumen or 10% abomasal fluid solutions did not affect subsequent gastrin secretion, and the C/A ratio was the same as for tissue incubated in control medium alone. The D/A ratios for tissues incubated in 10% rumen fluid, 10% abomasal fluid and 20% rumen fluid was not different from D/A ratio for tissues incubated in control medium alone.

Neither rumen nor abomasal contents affected non-specific binding (blanks) or maximum binding (zero) in the gastrin RIA. Since rumen and, to a lesser extent, abomasal contents markedly affected the values for blanks and zeros in the somatostatin assay, producing large values for both, the possible effects of gut contents on somatostatin secretion *in vitro* could not be studied.

4.2.3 Conclusions

The *in vitro* antral tissue test system used previously to study the effects of pharmacological agents on gastrin secretion also proved suitable to study the effects of rumen and abomasal contents. The effects of rumen and abomasal contents on gastrin secretion were reversible, as gastrin release in subsequent incubations was, in general, not affected by incubation with rumen or abomasal fluids. The presence of rumen or abomasal contents did not affect the non-specific binding nor gastrin antiserum binding in the gastrin RIA, but did interfere in the somatostatin RIA.

Lowering the osmolarity to 300mOsm.L^{-1} significantly increased gastrin release and a pH below 7.3 inhibited gastrin release, as was previously

reported by Lawton (1995). Test solutions must be adjusted to avoid these effects. The solutions were well buffered and the pH did not change during the incubations. A small increase in pH occurred in some solutions over a period of several hours, so the practice of adjusting the final pH no more than three hours before incubations was adopted.

Reversal of the inhibitory activity observed in rumen contents in the abomasal contents was not due to the presence of gastrin in the contents, which in all samples tested was less than 10pM. This concentration of gastrin was not enough to mask the large inhibitory effect of rumen contents, which reduced gastrin levels present in the basal medium after incubation by up to 400pM when compared to control (A) incubations. Gastrin in abomasal fluids added to basal medium for incubations was unlikely to have significantly affected estimates of the *in vitro* secretion response to abomasal contents, as the abomasal fluids were diluted at least 5-fold for incubation with tissue. Even if gastrin levels in abomasal fluids were 100pM, this would only equate to 20pM in the medium, while gastrin concentration in basal medium after incubation with tissue was between 150 and 500pM.

4.3 Serum gastrin and *in vitro* gastrin inhibitory activity of abomasal contents in parasitised sheep

4.3.1.1 Experimental overview

Gastrin inhibitory activity was determined in abomasal fluid collected from *O. circumcincta*-infected lambs. The lambs formed four groups according to the infection regimen:

Group 1 (control, or CTR): Parasite-naïve lambs (n=4) and which were injected daily with omeprazole between Days 24-27.

Group 2 (adult parasite infected or API): Infected with 20 000 *O. circumcincta* adult worms (n=6)

Group 3 (second larval parasite infected group, or LPI-2): Infected with 40 000 *O. circumcincta* L3 (n=4)

Group 4 (larval trickle infected, or LTI): Infected with 50 000 *O. circumcincta* L3, then from Day 35 with 10 000 L3 once a week for six weeks.

4.3.2 Methods

4.3.2.1 Animals

Groups 1-3: Fourteen Poll Dorset sheep (males and females) were reared indoors and kept helminth-free from birth. At approximately five months-of-age, all animals were fitted with abomasal cannulae at the junction of the antrum and the fundus under general anaesthesia, as previously described by Lawton *et al.* (1996). Animals were fed daily with 800g lucerne nuts and 200g aged hay. Water was supplied *ad libitum*. Four sheep (C1-4) were maintained uninfected as controls (CTR), six (A1-6) were infected with 20 000 *O. circumcincta* adult worms via the abomasal cannula (API) and four (1-4L) were infected with 40 000 *O. circumcincta* L3 by intraruminal intubation (LPI-2). API sheep were dosed orally on Day 12 post infection with 400μg.kg^{-1} ivermectin (Merial, New Zealand).

The control animals were injected intravenously with 20mg omeprazole (Astra Pharmaceuticals, NZ) on Day 24 and 40mg on Days 25-27.

In addition to the abomasal and blood sampling described below, fundic mucosal biopsies were collected for another study (Scott *et al.,* 2000). Two tissue biopsies were collected on each of 12 days during a 31 day experimental period in LPI and control animals, or 11 times during a 25 day experimental period in API animals.

Group 4: Eight Romney cross lambs (six months old, 30-35kg body weight, males and females) were raised parasite-free and surgically fitted with abomasal cannulae as above. The sheep were fed daily with 600g lucerne nuts and 1200g aged hay. They were infected with 50 000 *O. circumcincta* L3 intraruminally by tube, followed by a trickle infection with 10 000 L3 once a week for six weeks (on Days 35, 42, 49, 56, 63 and 70).

These sheep were being sampled by a fellow student, Ms. Sabine Przemeck, who was studying parasite resilience in a Massey sheep flock. As

part of that study, blood and abomasal fluid samples were collected twice daily before infection and until Day 24 post infection, thereafter twice a week. Data on abomasal pH and serum gastrin were kindly supplied by Ms Przemeck and are presented in Figure 4.13.

4.3.2.2 Blood and abomasal sampling

Groups 1-3: Blood and abomasal samples (for measurement of pH) were collected twice daily from three days prior to infection to Day 7 post infection, thereafter at variable intervals not exceeding three days until the end of the experiment. Blood was collected by jugular venepuncture into plain vacuated tubes, allowed to clot at room temperature, and centrifuged at 2000*g* for 20 minutes. The serum was separated and stored at -20°C for gastrin analysis.

Abomasal samples for *in vitro* analysis were collected on Days 2, (prior to infection), 3, 6, 7, 9, 15, 17, 20 and 22 (post infection) from sheep infected with adult worms; Days 2, 3, 6, 7, 9, 15, 17, 20, 22, 27 and 29 (all post infection) from sheep infected with L3; and on Days 6, 7, 9, 15, 17, 20, 22, 27 and 29 from control sheep.

Group 4: Abomasal fluid was collected for *in vitro* study on Days 13, 14 and 43. Samples were collected from seven sheep on the first four sampling days and from four sheep on the subsequent five occasions.

All samples were frozen for up to two months at -18°C before testing for inhibitory activity.

4.3.2.3 Abomasal pH and serum gastrin

Abomasal pH was measured using a PHM82 Standard pH Meter (Radiometer, Denmark) immediately after the abomasal sample was collected. Serum gastrin was estimated by RIA, as described in Appendix 1.

4.3.2.4 *In vitro* inhibitory activity of abomasal fluid

Abomasal samples were tested for their inhibitory activity on gastrin secretion at 10% final concentration using the *in vitro* method described in Section 2.1.1. Each sample was tested in two plates, on a total of 16 tissue pieces. On freezing, abomasal solutions separated into liquid and particulate

material layers. Only the liquid fraction was tested for inhibitory activity. Samples were tested in the order collected and all samples from one day were tested in the one experiment.

4.3.2.5 Gastrin "breakdown" *in vitro*

The possible breakdown of gastrin during the *in vitro* incubation such that it cannot be recognised by the antibody in the gastrin RIA, referred to as "breakdown," was assessed by incubation of test solutions with gastrin standard solutions under similar conditions to incubation of tissue pieces *in vitro.* Test solutions were added to four gastrin standards (50pM, two of 100pM, 200pM) to a final concentration of 10% and incubated at 37°C for 30 minutes, followed by incubation at 4°C for two to three hours. A control standard tube was set up for each concentration by adding the gastrin RIA diluent instead of the test solution. At the end of the incubations, the tubes were stored at -18°C for later gastrin estimation by RIA. The gastrin breakdown or "reduction ratio" was calculated by dividing the gastrin concentration in the standard/test mixture by the gastrin concentrations in the control standard solution. The mean±SEM was calculated for the four standards.

4.3.2.6 Statistical analyses

For abomasal pH and serum gastrin, the upper limit of the normal range was established for each animal and for the group as two SD above the mean of all pre-infection values. Any value above this level was considered elevated.

All *in vitro* data are presented as mean±SEM for each plate (eight pieces of tissue) unless otherwise indicated; "n" is the number of replicate experiments. The effects of test solutions were determined using UNIANOVA in SPSS version 9. All data were examined for normality using Shapiro-Wilks tests and for equality of variance using Levene's Homogeneity of Variance test. *Post hoc* comparisons of data groups when variances were equal was conducted using Tukey's HSD test for comparisons of all groups, or Dunnett's test when compared with control alone. When variances were unequal, Tamhane's T2 *post hoc* test was used. When the data were not normally distributed, Kruskal Wallis analysis in Graphpad Prism version 2.01, with Dunn's *post hoc* test was used.

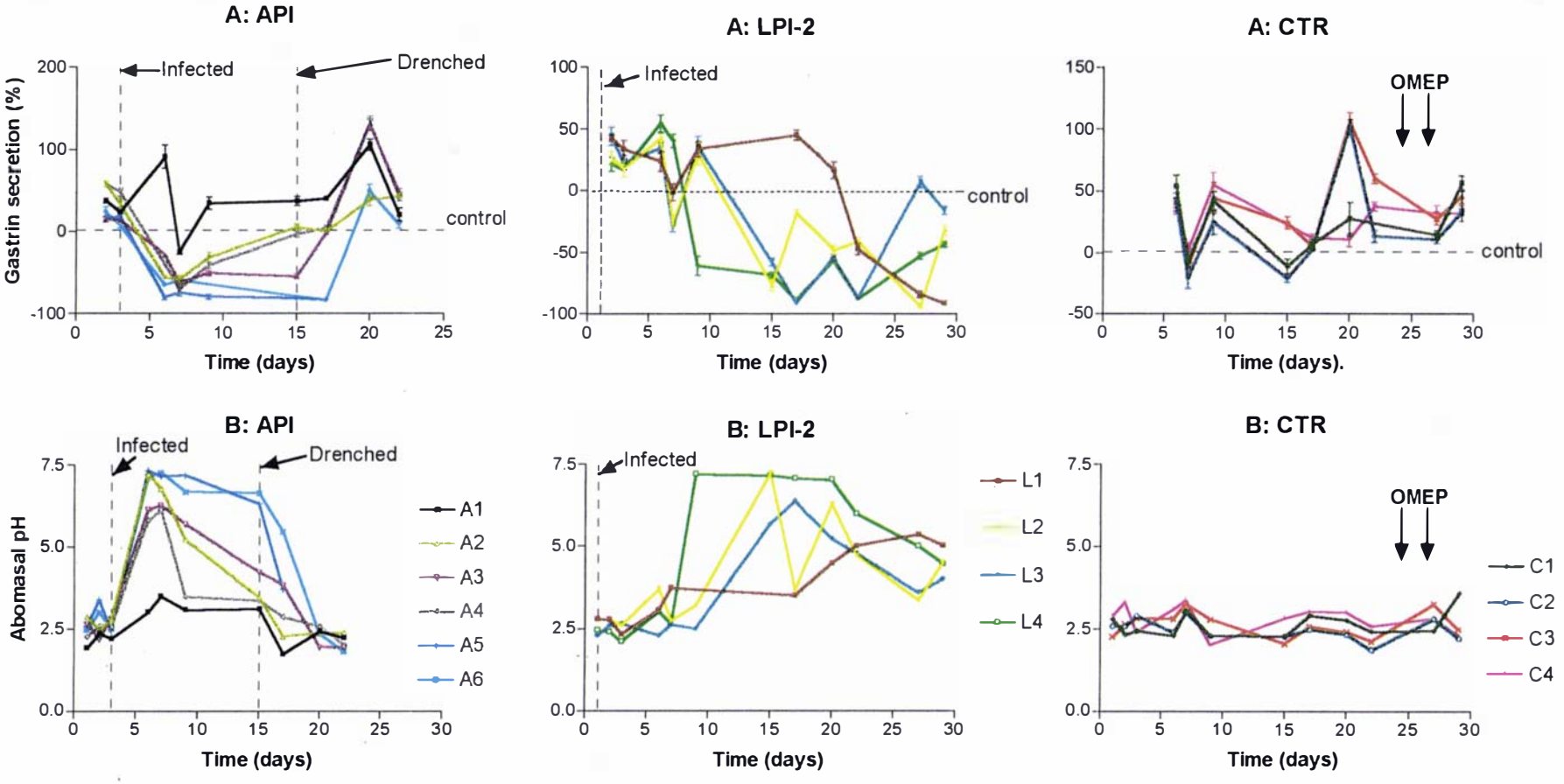

Figure 4.5. *In vivo* abomasal pH and *in vitro* gastrin secretion in response to abomasal contents from individual sheep infected with: (API) 20 000 adult *O. circumcincta*; (LPI-2) 40 000 L3 *O. circumcincta*; (CTR) uninfected. Effect on *in vitro* gastrin secretion, expressed as mean±SEM, is shown in A graphs, while abomasal pH is shown in B graphs. Arrows indicate infection times, drenching time in the adult infected group and the period of omeprazole treatment (OMEP) in uninfected animals .

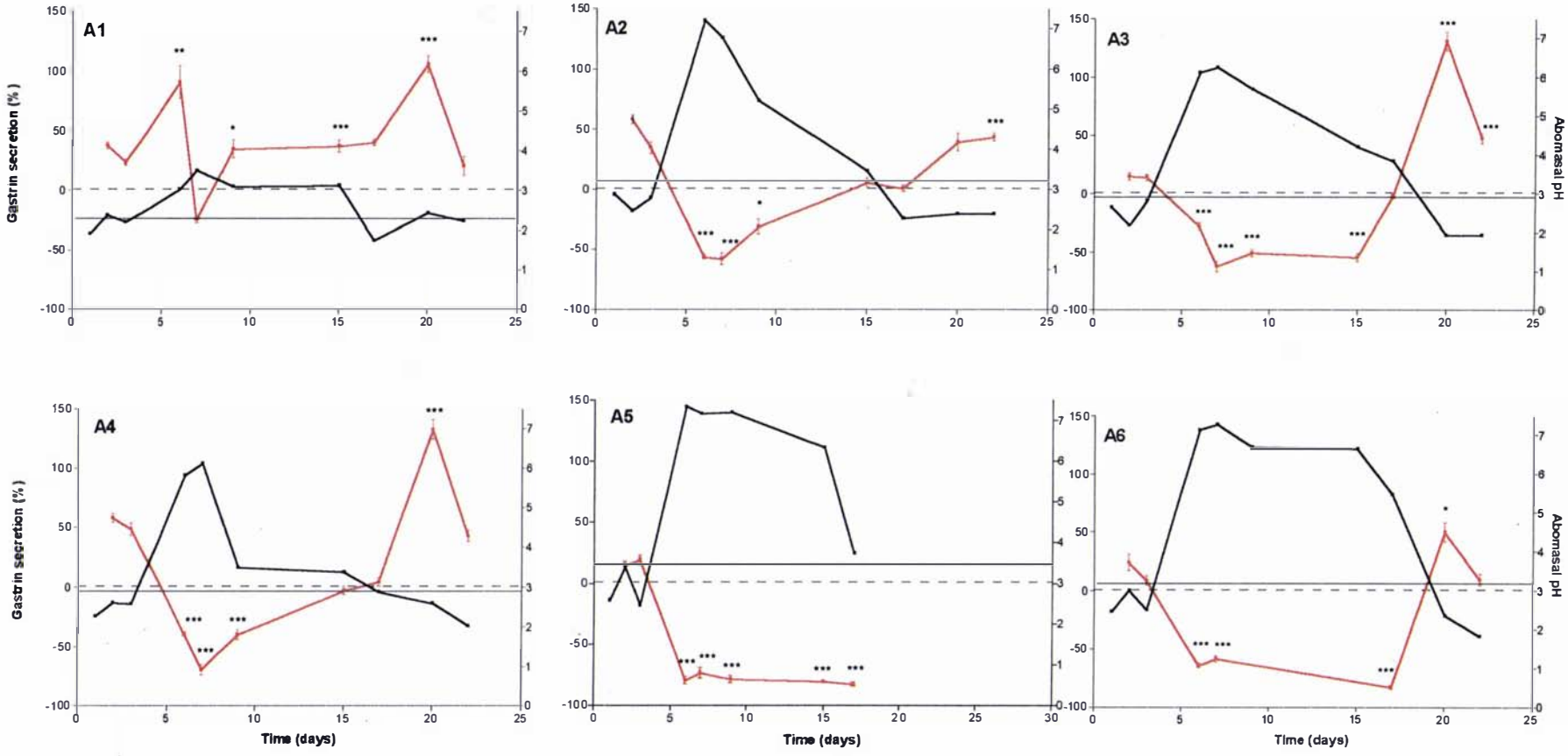

Figure 4.6. *In vivo* abomasal pH (━━) and *in vitro* gastrin secretion response (━━) to abomasal contents from six sheep infected with 20 000 adult *O. circumcincta.* Abomasal pH and *in vitro* gastrin secretion are expressed as mean±SEM, with the upper limit of normal pH values shown by the solid horizontal line and control gastrin secretion by the horizontal dotted line, *=p<0.05, **=p<0.01, ***=p<0.001, gastrin secretion significantly different from control.

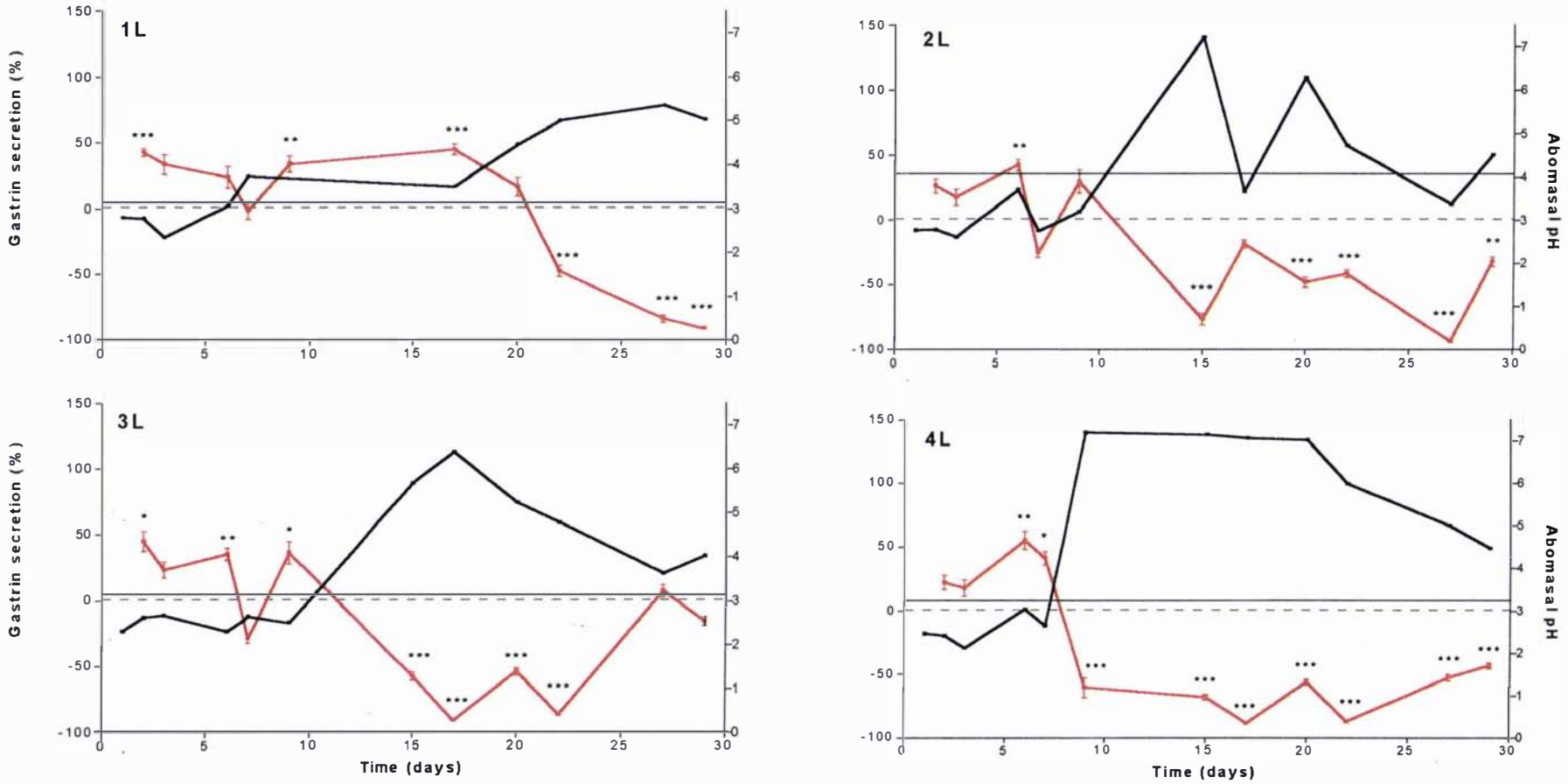

Figure 4.7. *In vivo* abomasal pH (—) and *in vitro* gastrin secretion response (—) to abomasal contents from four sheep infected with 40 000 L3 *O. circumcincta*. Abomasal pH and *in vitro* gastrin secretion are expressed as mean±SEM, with the upper limit of normal pH values shown by the solid horizontal line and control gastrin secretion by the horizontal dotted line, *=p<0.05, **=p<0.01, ***=p<0.001, gastrin secretion significantly different from control.

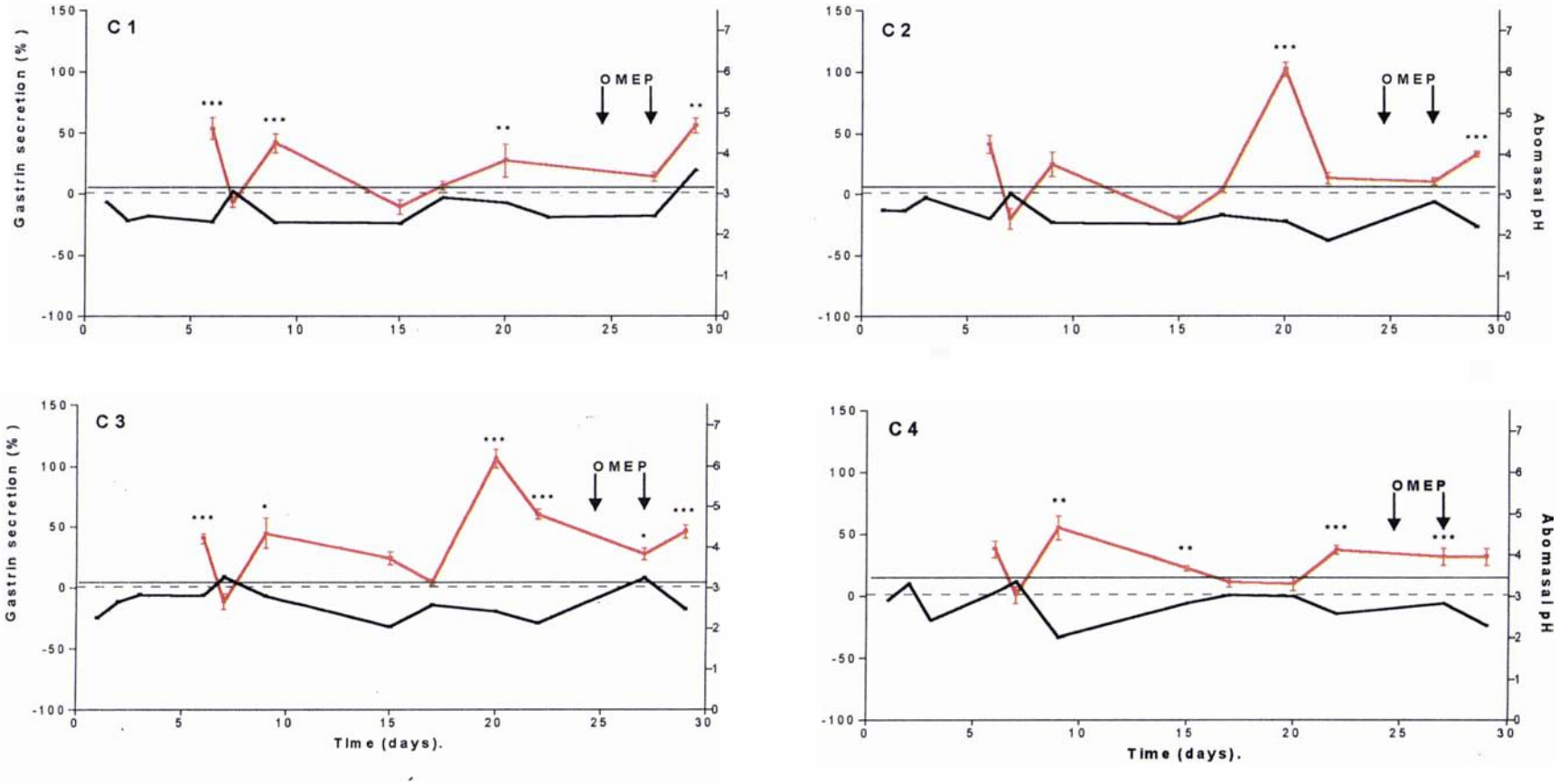

Figure 4.8. *In vivo* abomasal pH (—) and *in vitro* gastrin secretion response (—) to abomasal contents from four uninfected animals. Abomasal pH and *in vitro* gastrin secretion, expressed as mean±SEM are presented, with the upper limit of normal pH values shown by the solid horizontal line and control gastrin secretion by the horizontal dotted line, *=p<0.05, **=p<0.01, ***=p<0.001, gastrin secretion significantly different from control

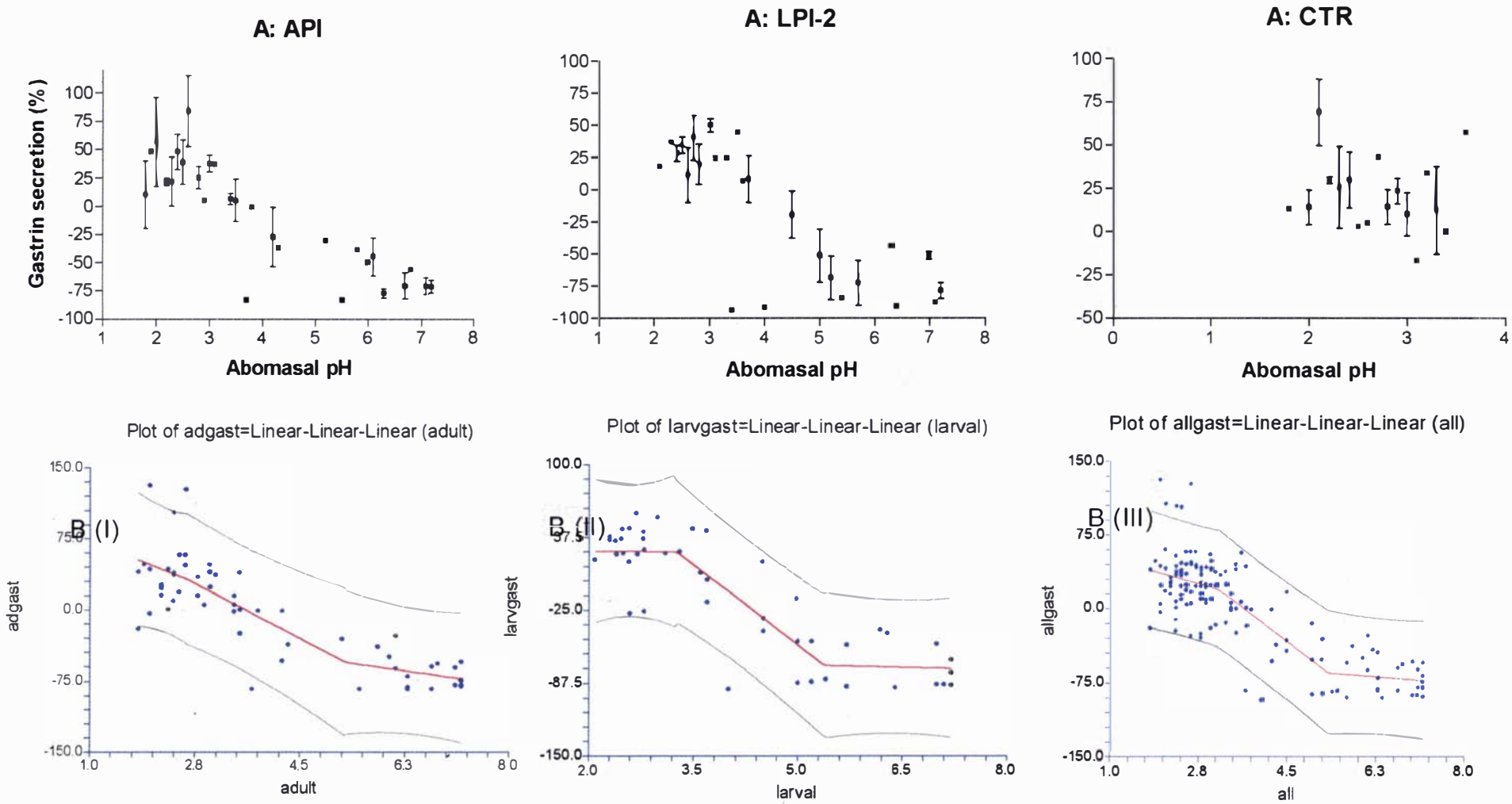

Figure 4.9. The relationship between abomasal pH and in vitro gastrin secretion in response to abomasal contents from API (infected with 20 000 adult O. circumcincta) LPI-2 (infected with 40 000 larval O. circumcincta) or CTR (uninfected) animals. Raw data is shown in A, and the linear-linear-linear model fitting API (I) , LPI-2 (II) and all data combined (III) shown in B. The model for API has joining points (mean±SEM) at pH 2.60±2.22 and pH 5.26±1.24, r2=0.69; for LPI-2 has joining points at pH 3.25±0.47 and pH 5.38±0.45, r2=0.75; for all data has joining points at pH3.15±0.44 and pH 5.31±0.48, rr= 0.67.

Correlation coefficients were calculated using SPSS version 9.0, using Pearson correlation for normally distributed data and Spearman correlation for data not normally distributed. Correlations between serum gastrin and abomasal pH were one-tailed analyses, while those between *in vitro* gastrin inhibitory activity and serum gastrin were two-tailed.

Data modelling of *in vitro* inhibitory activity of abomasal samples and sample pH was tested using linear-linear-linear model in NCSS97.

4.3.3 Results

4.3.3.1 *In vitro* gastrin inhibitory activity in abomasal contents

Abomasal contents from all parasitised sheep contained *in vitro* gastrin inhibitory activity on some days, but there was no inhibitory activity present in contents of the uninfected animals. There was a strong relationship between the presence of inhibitory activity and high abomasal pH, whereas samples with a low pH exhibited stimulatory activity. All samples which had a pH of 5 and above contained inhibitory activity. Four samples with pH below 5 also showed inhibitory activity: one with a pH of 3.4 and three with pH between 4.0 and 4.3. The relationship between *in vitro* activity and abomasal pH is illustrated in Figures 4.5 to 4.8. Data for all animals in the CTR, API and LPI-2 groups are summarised in Figure 4.5 and for individual animals in the API group in Figure 4.6, LPI-2 in Figure 4.7, and uninfected animals given omeprazole in Figure 4.8.

Three separate analyses showed that pooled data from all animals, from all API animals and from all LPI-2 animals fitted well to a linear-linear-linear model (Figure 4.9). The formula for the model is.

Gastrin secretion = $a1 + b1(pH)$ if $pH \leq J1$ (line 1)

$= a2 + b2(pH)$ if $J1 < pH \leq J2$ (line 2)

$= a3 + b3(pH)$ if $pH > J2$ (line 3).

Where J1 is the joining point between lines 1 and 2, and J2 is the joining point between lines 3 and 4. The a and b values were different for each group, as shown below:

Animal Number	Group	Correlation statistic	r^2 value	Correlation significance
1L	LPI-2	-0.72	0.52	0.05
2L	LPI-2	-0.46	0.21	ns
3L	LPI-2	-0.86	0.74	0.05
4L	LPI-2	-0.71	0.50	0.05
A1	API	-0.36	0.13	ns
A2	API	-0.93	0.86	0.05
A3	API	-0.75	0.56	0.05
A4	API	-0.85	0.72	0.05
A5	API	-0.84	0.71	0.05
A6	API	-0.91	0.83	0.05
C1	CTR	-0.26	0.07	ns
C2	CTR	-0.18	0.03	ns
C3	CTR	-0.45	0.20	ns
C4	CTR	-0.10	0.01	ns

Table 4.1. Correlation between serum gastrin concentration and *in vitro* gastrin secretion in response to abomasal contents from animals infected with either adult or L3 *O. circumcincta* or uninfected. Shown are the animal number, the infection group, API= infected with 20 000 adult *O. circumcincta*, LPI-2= infected with 40 000 L3 *O. circumcincta*, and CTR= uninfected animals. The correlation coefficient (correlation statistic) for a two-tailed Pearson correlation of serum gastrin and *in vitro* gastrin response to abomasal contents and the significance of this correlation are also shown.

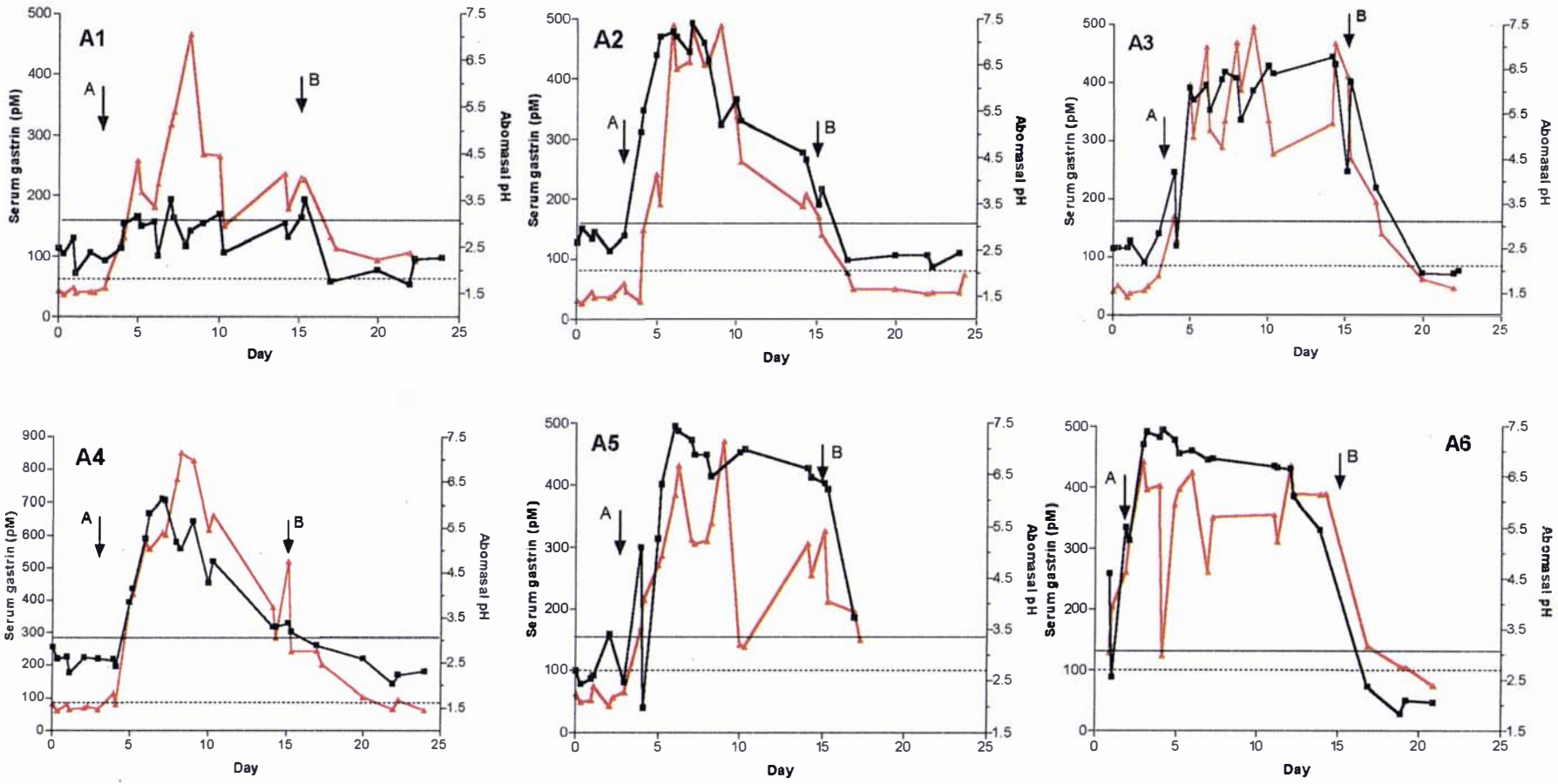

Figure 4.10. Abomasal pH (——) and serum gastrin (——) in six animals infected with 20 000 adult *O. circumcincta.* Infection times (arrow A) and the time of drenching with 400μg.kg^{-1} Ivermectin (arrow B) are shown, as well as the upper limits (mean + 2 SD) of normal pH (horizontal line) and serum gastrin (dotted line).

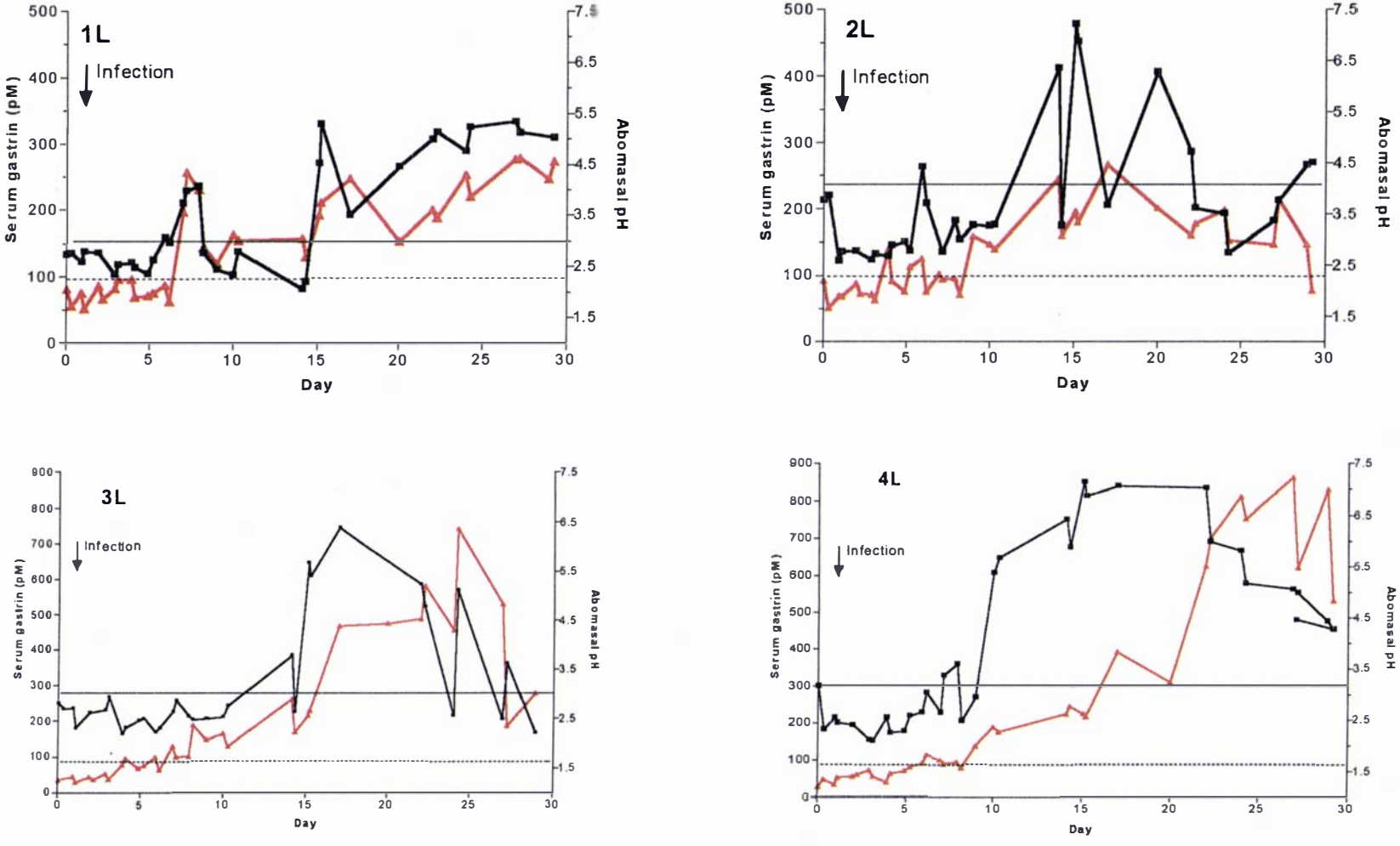

Figure 4.11. Abomasal pH (——) and serum gastrin (——) in four animals infected with 40 000 L3 *O. circumcincta.* The infection time (arrow A), is shown along with the upper limits (mean + 2.SD) of normal pH (horizontal line) and serum gastrin (dotted line).

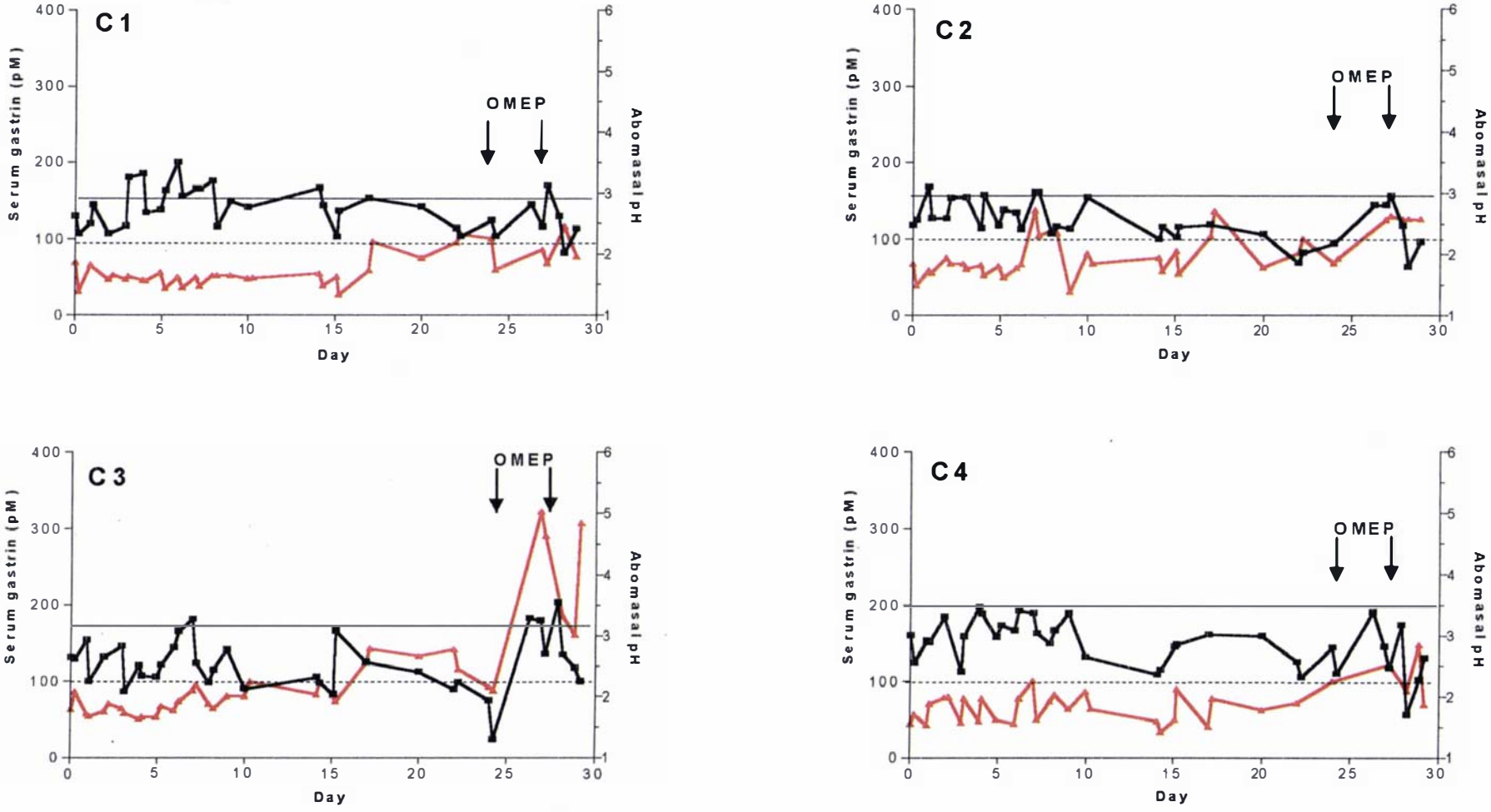

Figure 4.12. Abomasal pH (——) and serum gastrin (——) in four uninfected control animals. The upper limits of normal pH (horizontal line) and serum gastrin (dotted line) are shown. Arrows indicate time period of omeprazole treatment (OMEP)

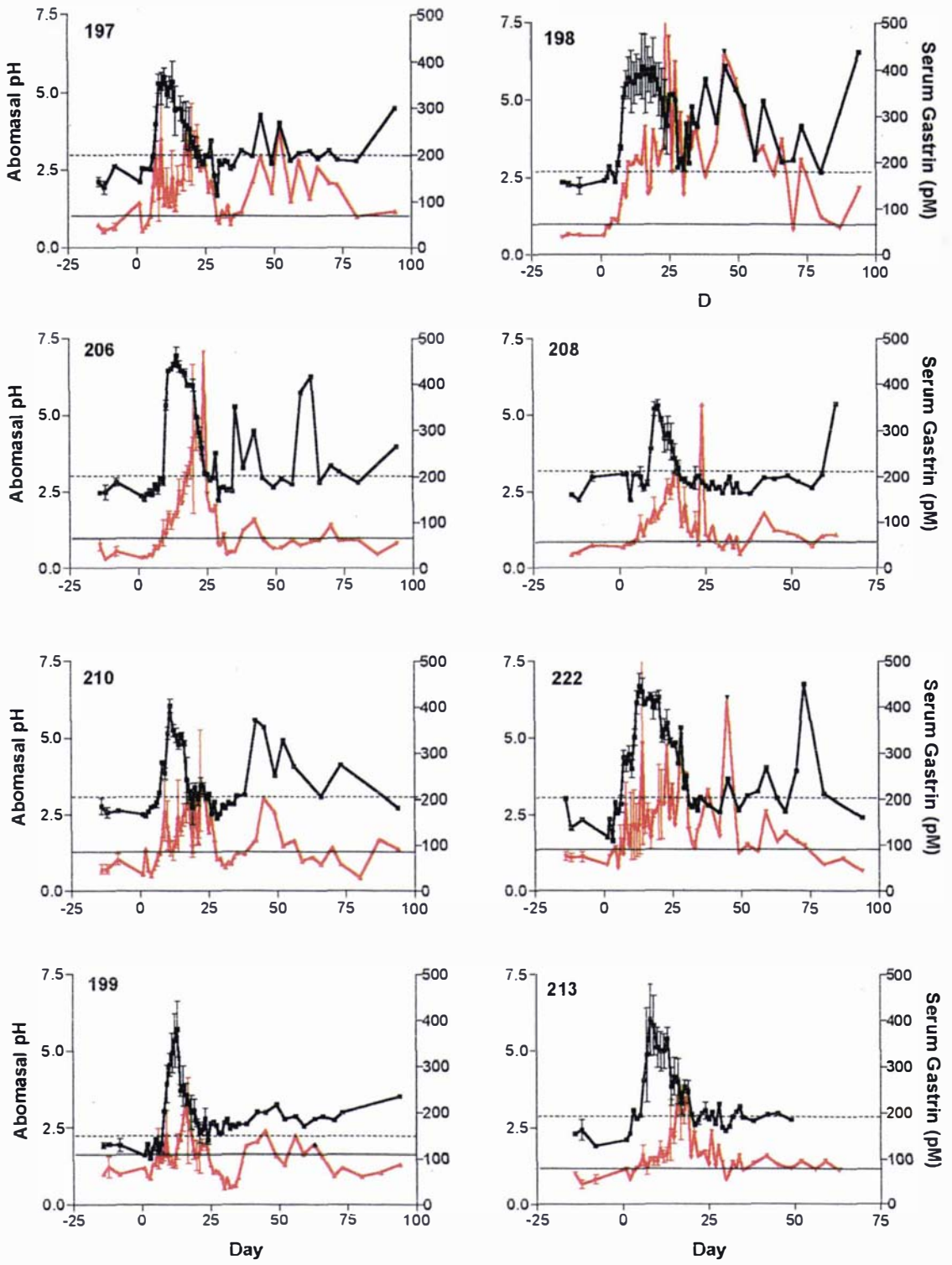

Figure 4.13. Serum gastrin (—) and abomasal pH (—) for eight sheep infected with 50 000 L3 *O. circumcincta* on Day 0 and trickle infected once weekly with 10 000 L3 *O. circumcincta* on Days 35, 42, 49, 56, 63 and 70. Horizontal dotted lines represent the upper limit of normal abomasal pH, while the solid line represents the upper limit of normal gastrin secretion. Gastrin and abomasal pH are expressed as mean±SEM when two samples were collected on one day.

API: a1 = 97.75; a2 = 118.64; a3 = -9.48; b1 = -25.07; b2 = -33.11; b3=-8.75; J1 = 2.60, J2 = 5.26; r^2=0.69

LPI-2: a1 = 66.08; a2= 142.75; a3 = -43.50; b1 = -14-69; b2 =-39.01; b3 = -3.99; J1 = 3.26; J2 = 5.38; r^2=0.75

All data: a1 = 66.08; a2 = 142.74; a3 = -43,50; b1 = -14.68; b2 = -39.02; b3 = -4.00;J1= 3.15, J2= 5.32; r^2=0.67.

A negative correlation between serum gastrin concentration and *in vitro* gastrin secretion in response to abomasal contents was observed for three of the four LPI-2 animals and five of the six API animals (Table 4.1). Thus, high amounts of *in vitro* gastrin inhibitory activity in abomasal contents coincided with high serum gastrin levels. There was no significant correlation between serum gastrin and *in vitro* gastrin secretion in response to abomasal contents in control animals.

Three samples were taken from each sheep in the LTI group on Days 13 and 14, when abomasal pH was highest. On Day 43, when the pH was returning to normal, samples were taken from all animals except Sheep 199 and 197. Significant inhibitory activity was observed only in samples with a pH of 4.8 and over.

4.3.3.2 Relationship of serum gastrin to abomasal pH

Serum gastrin levels and abomasal pH for individual animals are shown in Figures 4.10 (API), 4.11 (LPI-2), 4.12 (CTR) and 4.13 (LTI). Parasitised sheep all had periods of hypergastrinaemia and elevated abomasal pH. Administration of omeprazole had inconsistent effects on abomasal pH and serum gastrin concentration, causing an increase in both briefly in C3, an increase in abomasal pH alone in C2, but no effect in other animals. There were no significant correlations for individual animals or for pooled data from all animals during omeprazole. For C3, where omeprazole did increase both gastrin and abomasal pH, correlation was close to significance (p=0.051, r=0.73), but correlation analysis involved only six data points.

Animal	Group	Correlation test	Correlation statistic	r^2 values	Correlation significance
C1	CTR	Pearson	r=0.34	0.12	0.05
C2	CTR	Pearson	r=0.10	0.01	ns
C3	CTR	Spearman	ρ=0.10	0.01	ns
C4	CTR	Pearson	r=0.27	0.07	ns
1L	LPI-2	Spearman	ρ=0.66	0.44	0.01
2L	LPI-2	Spearman	ρ=0.54	0.29	0.01
3L	LPI-2	Spearman	ρ=0.44	0.19	0.01
4L	LPI-2	Spearman	ρ=0.78	0.61	0.001
A1	API	Pearson	r=0.65	0.42	0.001
A2	API	Spearman	ρ=0.73	0.53	0.001
A3	API	Spearman	ρ=0.76	0.58	0.001
A4	API	Spearman	ρ=0.89	0.79	0.001
A5	API	Spearman	ρ=0.73	0.53	0.001
A6	API	Spearman	ρ=0.74	0.55	0.001
197	LTI	Spearman	ρ=0.43	0.18	0.01
198	LTI	Spearman	ρ=0.44	0.19	0.01
206	LTI	Spearman	ρ=0.70	0.49	0.01
208	LTI	Spearman	ρ=0.62	0.38	0.01
210	LTI	Spearman	ρ=0.36	0.13	0.01
222	LTI	Spearman	ρ=0.43	0.18	0.01
199	LTI	Spearman	ρ=0.41	0.17	0.01
213	LTI	Spearman	ρ=0.54	0.29	0.01

Table 4.2. Correlation between abomasal pH and serum gastrin levels in *O. circumcincta* infected sheep and controls. The infection regimens used were: CTR = no infection; LPI-2 = 40 000 L3 *O. circumcincta* larvae intraruminally; API = 20 000 Adult *O. circumcincta* into abomasum; LTI= infected with 50 000 L3 *O. circumcincta* and after 35 days with 10 000 L3 *O. circumcincta* once weekly for 6 weeks. The correlation tests conducted were Pearson correlation for normally distributed data or Spearman correlation non-normal distribution. The correlation coefficient statistic and the statistical significance of the relationship are also shown, with ns=not significant, 0.05=p<0.05, 0.01=p<0.01, 0.001= p<0.001. All correlations were one-tailed.

Animal number	Day 13			Day 14			Day 43		
	Sample pH	secretion	"breakdown"	Sample pH	secretion	"breakdown"	Sample pH	secretion	"breakdown"
199	4.58	-32±8%	0.82±0.01	3.65	+2±12%	0.88±0.07	-	-	-
198	5.14	-66±4%***	0.87±0.05	5.14	-77±1%***	1.12±0.07	6.67	-77±3%**	1.04±0.09
197	4.60	-34±4%	0.93±0.11	3.80	+23±15%	1.13±0.03	-	-	-
222	6.47	-85±3%***	1.07±0.14	6.20	-87±2%***	0.99±0.10	2.34	+25±16%	0.89±0.08
210	4.81	-51±6%**	1.10±0.08	4.62	+26±24%	0.56±0.07	3.65	+22±9%	1.14±0.15
213	4.93	-55±4%**	0.65±0.06	3.28	+10±10%	0.96±0.05	2.90	+8±15%	1.11±0.11
206	6.51	-85+1%***	0.80±0.08	6.79	-58±6%***	1.08±0.04	3.00	+77±10%***	1.10±0.07
208	3.82	+4±8%	1.07±0.04	3.91	+10±10%	1.00±0.09	2.84	+19±15%	1.09±0.06

Table 4.3. Gastrin "breakdown" activity and *in vitro* gastrin response to abomasal contents from sheep infected with L3 *O. circumcincta*. Shown are the sheep from which the samples came (animal number), the days on which the samples were taken, the sample pH (pH), the effect on *in vitro* gastrin secretion and whether secretion was different from control (*in vitro*, **=$p<0.01$, ***=$p<0.001$), and the reduction of gastrin levels by incubation of sample with gastrin standard solutions, expressed as a ratio (breakdown). Animals were infected with 50 000 L3 *O. circumcincta* on Day 0, and then from Day 35 with 10 000 *O. circumcincta* once weekly for 6 weeks,

Periods when serum gastrin was depressed in spite of elevated abomasal pH were seen in Sheep 4L in the LPI-2 group, briefly in Sheep A5 and for one sample in Sheep A6 of the API group, and in Sheep 199 and 210 in the LTI group. These periods of low serum gastrin appeared unrelated to *in vitro* gastrin inhibitory activity. High amounts of inhibitory activity in the abomasal contents were observed with elevated abomasal pH and low serum gastrin. However, the opposite situation also occurred frequently, where high amounts of inhibitory activity coincided with high abomasal pH and serum gastrin.

Abomasal pH and serum gastrin were closely correlated in the API group (r or ρ between 0.65 and 0.89; mean 0.75), whereas the correlation was less stringent for the LPI-2 group (r or ρ between 0.44 and 0.78; mean 0.61) and the LTI group (r or ρ between 0.36 and 0.70; mean 0.49) and there was no correlation for the CTR group (r or ρ between 0.10 and 0.34; mean 0.20). Correlation values for individual sheep are shown in Table 4.2. These data compare with the correlation coefficients for the sheep infected with a predominantly antral strain of *O. circumcincta* shown in Figure 3.10 (r or ρ between 0.12 and 0.52; mean 0.32).

In LTI sheep, samples were taken before and after feeding, and on some days, small increases in serum gastrin concentration and decreased abomasal pH were observed after feeding.

4.3.3.3 Gastrin "breakdown" by abomasal contents

Gastrin "breakdown" from standard solutions by abomasal contents from sheep in the LTI group was evident only in two samples (Table 4.3). This activity appeared unrelated to abomasal pH, or to the *in vitro* release of gastrin by tissue (Table 4.3). Overall, the inhibitory activity in abomasal contents from the LPI-2 sheep could not be attributed to "breakdown" of gastrin after secretion in the *in vitro* test.

4.4 Discussion

Experiments presented in this Chapter have shown that when abomasal pH is elevated in sheep parasitised with *O. circumcincta*, there is activity present in the abomasal contents, which can inhibit *in vitro* gastrin secretion. This was not due to interference with the gastrin assay, nor "breakdown" of gastrin during the *in vitro* incubation. The presence of inhibitory activity appeared to be primarily determined by abomasal pH, but did not appear to affect serum gastrin concentrations during *O. circumcincta* infection.

4.4.1.1 Inhibitory activity in abomasal contents

Abomasal contents from sheep infected with either larval or adult *O. circumcincta* contained inhibitory activity when abomasal pH rose to levels above 4.5-5. The appearance of inhibitory activity occurred later in sheep infected with larval *O. circumcincta* than in sheep infected with adult *O. circumcincta*, coinciding in both cases with an increase in abomasal pH. In animals infected with adult *O. circumcincta*, inhibitory activity disappeared with the resumption of normal acid secretion after drenching, and was never apparent in control animals (Figure 4.5, CTR A and B). The appearance of inhibitory activity may have been masked by stimulatory activity present in abomasal contents, perhaps caused by amino acids or dietary ammonia, as these have been shown to stimulate gastrin secretion (Lichtenberger *et al.,* 1982a, b ; Dial *et al.,* 1986, 1991). In the majority of abomasal samples with low pH, however, any increases in gastrin secretion were not statistically significant ($p>0.05$), and so the presence of a stimulant masking inhibitory activity is unlikely.

The model that best fitted the relationship between the pH of the abomasal contents and its gastrin inhibitory activity was a linear-linear-linear one. This model predicts that up to a certain pH (J1) there is no inhibitory activity, then between J1 and J2 the inhibitory activity increases until J2, beyond which inhibitory activity is maximal. This three-line pattern was particularly evident when modelling data from all animals and from LPT-2 animals. For the API group J1, the point where the presence of inhibitory activity starts to increase, was poorly defined (Figure 4.10). This was probably due to the lack

of data between pH3 and pH5 where intermediate inhibitory responses would be expected. Data from the LPI-2 group showed a more distinct J1 point at 3.25±0.48, with data from this group probably defining the J1 point in the combined data for all sheep. In both API and LPI groups, J2 was between pH5.2-5.5, and this point was well conserved in the plot of all data. No model was found which fitted data from the control animals. Overall, the data suggest that the inhibitory activity started to appear in abomasal solutions at pH3.25±0.5, gradually increased between pH3.5-5 and was near maximal at 5.3±0.5. Factors other than pH may also affect the levels of inhibitory activity present in the abomasum, such as the length of period of abomasal pH elevation. *In vitro* tests also showed different amounts of activity at the same sample pH, even at pH5.5 and greater, suggesting that there may be different compounds with different potencies in causing the inhibitory effect.

A possible origin of the inhibitory activity is the rumen. All rumen samples from all animal groups contained inhibitory activity, regardless of the infection state of the animal. In the experiments described in the Chapter 3, bacterial survival in the abomasum was maximal at pH3.5 and over. This point is very similar to J1, where modelling predicted that inhibitory activity first started to appear. The pH levels at which inhibitory activity has significant effect on *in vitro* gastrin secretion are above this, around pH4.5-5, but such levels are still below those suitable for rumen bacteria proliferation (Joblin, personal communication). It therefore seems unlikely that an increase in metabolic activity by bacteria in the abomasum significantly increases levels of inhibitory activity. More likely is the survival of a compound originating from the rumen, which is usually destroyed by low abomasal pH.

Incubation of abomasal contents with gastrin standard solutions showed that in the majority of cases, this inhibitory activity could not be explained by breakdown of released gastrin.

4.4.1.2 Serum gastrin, abomasal pH and abomasal inhibitory factor

Although an activity which can inhibit gastrin secretion, *in vitro* at least, does exist in the abomasal contents when pH is elevated above 3.3, it appears to have little effect on serum gastrin levels during abomasal parasitism.

Sheep in the API group showed a close correlation between abomasal pH and serum gastrin (Table 4.1), similar to results reported by Fox *et al.* (1993) for calves infected with *O. ostertagi.* This was despite large amounts of *in vitro* inhibitory activity noted in the abomasal contents at pH5 and higher.

The correlation between serum gastrin and abomasal pH was poorer in sheep infected with larval *O. circumcincta*. Two larval infected animals (3L and 4L) exhibited a lag in hypergastrinaemia behind hypoacidity as reported in LPI-1 experiment in Chapter 3. These animals did show the greatest and most consistent inhibitory response of the LPI-2 animals. These levels were not, however, greater than those observed in sheep infected with adult *O. circumcincta*, in which abomasal pH and serum gastrin followed each other closely. Thus, the weaker correlation between serum gastrin and abomasal pH in sheep infected with larval *O. circumcincta* was not likely to have been due to abomasal inhibitory activity.

In the LTI group, the correlation between serum gastrin and abomasal pH was again poorer than that for the API group. Three (197, 199 and 210) of the eight sheep exhibited a decrease in gastrin secretion during high abomasal pH, as described by Lawton *et al.* (1996). The presence of inhibitor in the abomasal contents alone is not enough to cause this effect though, as inhibitory activity is also present in solutions obtained from animals where gastrin secretion is significantly elevated.

In general, the r^2 values for correlations for LPI-1, LPI-2 and LTI animals were spread between 0.10 to 0.30. Of the 19 animals in LPI-1, LPI-1 and LTI, only one exhibited a correlation with r^2 values above 0.50, and three others had r^2 values above 0.4. Thus, in general larval infection resulted in a much poorer correlation between abomasal pH and serum gastrin than in the API group, and much lower than correlation values reported for cattle infected with *O. ostertagi* (Fox *et al.*, 1993). The data from the present experiments suggest that factors other than abomasal pH have significant effects on gastrin secretion in sheep during a larval *O. circumcincta* infection, though abomasal pH plays a greater role in elevation of gastrin secretion in the presence of adult worms, where less penetrative damage of the mucosa is expected. There was no correlation

between abomasal pH and serum gastrin in control animals, similar to results in calves (Fox *et al.,* 1993).

It may be necessary for bacteria to have a close association with the G cell in order for the inhibitory effect to affect gastrin release. G cells have an apical border which has a tuft of microvilli that extend into the lumen of the pyloric gland (Creutzfeldt *et al.,* 1971; Bunnett, 1984), and thus may be responsive to luminal chemicals such as amino acids, which in Chapter 2 were shown to stimulate gastrin release. It is therefore possible that G cells could respond to the presence of a gastrin inhibitor in the abomasal contents. Lawton (1995) reported bacterial production of gastrin inhibitory activity when incubated in basal medium at 37°C for 24 to 48 hours. If a bacterial population developed in the pyloric glands, in a similar fashion to the bacterial colonisation of fundic pits in some calves with chronic diarrhoea (Gunning, 2000), and the bacteria produced gastrin inhibitory activity, inhibition of the G cell may be possible due to the close proximity of bacteria to the G cell. This may have caused the depression in gastrin secretion observed in some sheep when abomasal pH rose to 5.5 and over (Lawton *et al.,* 1996). However, in experiments presented here, abomasal contents were shown to contain enough inhibitory activity to affect *in vitro* gastrin secretion when diluted 10-fold. If the G cell is responsive to luminal constituents, the hypoacidic abomasal contents tested in experiments presented here would be expected to contain enough inhibitory activity to affect gastrin secretion. It is thus unclear why the inhibitory activity shown to exist in abomasal contents did not affect gastrin secretion.

There is no clear indication that inhibitory activity in the abomasum was the cause of abnormal fluctuations in gastrin secretion reported by Lawton *et al.* (1996) in sheep parasitised with *O. circumcincta.* If such an inhibitor does have an effect on gastrin secretion as Lawton (1995) suggested, then there must be some as yet unknown condition that facilitates this action.

In the next chapter, the generation of inhibitory activity during incubation of abomasal and rumen contents in basal medium, and properties of the inhibitory activity present in rumen and incubate solutions are examined further.

4.4.2 Summary

Abomasal contents do contain a factor which can inhibit gastrin secretion *in vitro.* Modelling of data obtained from both adult and larval *O. circumcincta* infection in sheep show the *in vitro* gastrin inhibitory factor probably starts to survive at around pH3.2, but its effects are not significant until pH4.5-5 and are near maximal at pH5.3 and over. The negative correlation between *in vitro* gastrin secretion in response to abomasal contents and serum gastrin concentrations (r values between -0.36 and -0.93) suggest that inhibitory activity does not affect gastrin secretion *in vivo* during abomasal parasitism.

There is a strong correlation between serum gastrin and abomasal pH in animals infected with adult *O. circumcincta* (r^2 values for correlations between 0.42 and 0.79), but this correlation is weaker in animals infected with larvae (r^2 between 0.19 and 0.61). Larval infection, which has greater degree of mucosal involvement, may activate other factors that affect gastrin secretion, such as inflammation.

The role, if any, of this inhibitory activity in the control of gastrin secretion in the parasitised animal *in vivo* remains to be determined. Results presented here from a number of animals suggest that luminal involvement in gastrin secretion during abomasal parasitism is minimal. Most effects are most likely explained by increased abomasal pH, tissue pathology and possibly inflammation. The role of inflammatory mediators in controlling gastrin secretion has not been defined and may explain the why hypergastrinaemia is so marked. A factor that can inhibit *in vitro* gastrin secretion is present at high pH in the abomasal contents of parasitised sheep, but its effects on *in vivo* gastrin secretion appear to be minimal.

CHAPTER 5

PROPERTIES OF THE INHIBITOR OF *IN VITRO* GASTRIN SECRETION

5.1 Introduction

Abomasal contents of parasitised sheep consistently contained an inhibitor of *in vitro* gastrin secretion in samples in which pH exceeded 4.5 (Chapter 4). The rumen may be the source of the inhibitory activity, which then survives in the high pH of the parasitised abomasum. Lawton (1995) reported that incubation of abomasal contents also generated gastrin inhibitory activity. The development of this inhibitory activity occurred over a 24-48 hour incubation period in aerobic conditions and was inhibited by the presence of antibiotics, suggesting that it was of bacterial origin (Lawton, 1995). The existence and properties of gastrin inhibitory activity in incubates of abomasal and rumen fluid, and in rumen fluid itself were examined in experiments presented in this chapter.

The aims of the experiments in this chapter were to define whether the rumen fluid contained *in vitro* gastrin inhibitory activity and to identify under what conditions inhibitory activity was produced by abomasal and rumen incubates. The properties of the inhibitory activity were also examined. Specifically, how much of the inhibitory activity in both incubates and rumen fluids could be explained by gastrin "breakdown," whether activity was removed when bacteria were removed or inactivated, and the sensitivity of the inhibitor to acidity and temperature were determined.

5.2 Methods

5.2.1 Abomasal and rumen fluid

Abomasal fluid was collected from the abomasal cannulae of four sheep (ABO-1 to ABO-4) which were housed indoors in metabolism crates. Another six samples, which were used to generate abomasal incubates, were obtained at necropsy from a further six sheep which had been orally dosed with 0.7 $mg.kg^{-1}$ Ivermectin (Ivomec, Merial New Zealand Ltd), when brought indoors seven days before sampling. All animals were fed lucerne chaff *ad libitum* and had free access to water.

Rumen fluid (RUM) was obtained from three sources:

(a) rumen contents were collected by intubation six days prior to infection and on Days 9 and 22 after infection from 14 sheep (numbers 1L to 4L, API1 to API6 and C1 to C4) described in Chapter 4 (Section 4.3.2).

(b) seven rumen samples were obtained *post-mortem* from animals fed on lucerne chaff; of which five were tested for effects on gastrin secretion from the *in vitro* tissue preparation (RUM-1 to RUM-5), and two only for production of incubates.

(c) 33 samples were collected from sheep with rumen fistulae grazing at pasture, 20 of which (RUM-6 to RUM-26) were tested for effects on gastrin secretion from the *in vitro* tissue preparation, while the other 13 were used only to generate rumen incubates.

Abomasal and rumen contents were squeezed through a double layer of cheesecloth, producing a thick liquid and removing large plant particulate matter. Abomasal and rumen fluids were used within 24 hours, either to generate incubates or tested for effects on gastrin secretion from the *in vitro* tissue preparation. Samples for repeat testing were stored at -18°C for up to three months.

In some cases, further abomasal and rumen fluid was collected only for production of incubates. Details of such rumen fluids are given below in a description of the incubate solution.

5.2.2 Abomasal and rumen incubates

Seven abomasal and 23 rumen fluid samples were used to generate microbial cultures in liquid medium (basal medium based on HBSS, composition in Section 2.2.1.1).

Abomasal and rumen solutions were diluted 50- to 500-fold in 500mL or 1L of basal medium and incubated for up to 96 hours. Specific details for each incubate are given below.

AI-1 (Abomasal incubate 1). An abomasal sample obtained *post-mortem* was diluted 100-fold and incubated at 37°C in aerobic conditions for 22 hours in: normal basal medium; basal medium without BSA; normal basal medium maintained at pH7.20-7.40 throughout the incubation. As a control, basal medium without abomasal contents was also incubated for the same time period. Samples of 20mL of the incubate solutions were obtained after 0, 2, 3, 4, 5, 6, 7, 8, 12, 14, 16, 18, 20 and 22 hours of incubation.

AI-2 (Abomasal incubate 2). The same procedure was used as for ABI-1, except that samples were obtained after 0, 2, 4, 5, 6, 8, 10. 12, 29 and 48 hours of incubation.

AI-3 (Abomasal incubate 3). Abomasal sample obtained *post mortem* was diluted 50-fold and incubated at 37°C for 96 hours in: normal basal medium; basal medium without BSA; basal medium without glucose. As a control basal medium was incubated without abomasal contests for the same period of time. Samples of 20mL of the incubate solutions were obtained after 0, 3, 4, 5, 6, 7, 8, 9 and 96 hours of incubation.

AI-4 and AI-5 (Abomasal incubates 4 and 5). Abomasal samples obtained *post-mortem* were diluted 500-fold and incubated at 37°C for 48 hours in: normal basal medium; basal medium without BSA; basal medium without glucose; basal medium with $1mL.L^{-1}$ Penicillin (5000 units)/Streptomycin (5mg) (GIBCO BRL, Life Technologies Inc., USA) and $25mg.L^{-1}$ Kanamycin (K1876, Sigma Co., St Louis, USA); basal medium with $1mL.L^{-1}$ Penicillin (5000 units)/Streptomycin (5mg) liquid (GIBCO BRL, Life Technologies Inc., USA); basal medium with $20mg.L^{-1}$ Neomycin (N1876 Sigma Co., USA). Samples of 20mL of the incubate solutions were obtained after 0, 2, 4, 8, 24 and 48 hours of incubation.

AI-6 and AI-7 (Abomasal incubate 6 and 7). Abomasal samples obtained *post-mortem* from parasitised sheep and diluted 100-fold in normal basal medium and incubated at 37°C for 30 hours. Samples of 50mL were taken after 0, 1.5, 3, 4.5, 6, 7, 9, 12, 15, 19.5, 21, 24, and 30 hours of incubation. The pH of the abomasal sample for AI-6 was 2.65 prior to incubation, while the sample that of AI-7 was 4.2 but adjusted to pH 2.40 with 1M HCl prior to incubation.

AI-8, AI-9 and AI-10 (abomasal incubates 8, 9 and 10). Abomasal samples from parasitised sheep were diluted 500-fold in basal medium and incubated at 37°C for 48 hours. Abomasal samples before incubation had pH of 6.67 for AI-8, 3.00 for AI-9 and 3.65 for AI-10.

RI-1 and RI-2 (Rumen incubates 1 and 2). Rumen samples obtained from the same sheep as AI-4 and AI-5 respectively. Rumen fluids were obtained *post-mortem* and were diluted 500-fold in basal medium and incubated from 48 hours, with 20mL samples taken from the incubate solutions after 0, 2, 4, 8, 24 and 48 hours of incubation.

RI-3 to RI-8 (Rumen incubates 3 to 8). Rumen samples from six pasture-fed animals were diluted 500-fold and incubated at 37°C for 40 hours in normal basal medium. Samples of 20mL of the incubate solutions were obtained after 0, 20, 26 and 40 hours of incubation.

RI-9 to RI-14 (Rumen incubates 9 to 14). As for RI-3 to RI-8, except samples were collected after 0, 14, 18, 48 and 70 hours of incubation.

R-15 to RI-20 (Rumen incubates 15 to 20). As for RI-3 to RI-8, except samples were collected after 0, 12, 40, 64 and 80 hours of incubation.

RI-21 to RI-23 (Rumen incubates 21 to 23). As for RI-3 to RI-8 except samples were collected after 0, 12, 24 and 48 hours of incubation.

RI-24 to RI 27 (Rumen incubates 24 to 27). As for RI-3 to RI-8 except samples were collected only after 38 hours incubation.

RI-28 and RI-29 (Rumen incubates 28 and 29). As for RI-3 to RI-8 except samples were collected only after 48 hours incubation.

5.2.3 Bacterial counts

5.2.3.1 Preparation of basal medium agar plates

Plates were prepared from agar (1.5% (w/v) Bacto Agar (Fort Richard, USA) in sterile basal medium (composition described in Section 2.2.1.1). The agar medium was made by mixing two solutions: one containing the buffers and salts was autoclaved and the second solution containing glucose and BSA was filtered. The autoclaved solution (HBSS/agar solution) contained 15g Bacto-Agar, 5.66g HEPES, 0.35g bicarbonate and a 1L sachet of HBSS powder in 900mL double distilled H_2O, pH7.40±0.02. This solution was autoclaved for 20 minutes and then placed in a 37°C water bath to cool. The filtered solution contained 2.5g BSA and 1g glucose in 100mL. This solution was filtered through a N_2 pressurised filter system which passes solutions though a dual membrane, the first a prefilter (Millipore, AW0314250) and the second a 0.22µm hydrophilic filter (Millipore, GVWP142 50). When the autoclaved solution had cooled to 37°C, it was combined with the filtered BSA/glucose solution, and then poured into petri dishes in a laminar flow hood, allowed to set, covered and stored inverted at 4°C until used.

5.2.3.2 Bacterial enumeration

Bacteria in abomasal incubates ABI-1 and ABI-2 were enumerated aerobically on basal medium-agar plates. The sterile medium for dilution was prepared in the same manner as the preparation of basal medium agar plates described in 5.3.2.1: a salt solution containing 5.66g HEPES, 0.35g bicarbonate and a 1L sachet of HBSS powder in 900mL double distilled H_2O, pH 7.40±0.02 was autoclaved for 20 minutes, then cooled to 37°C and combined with a 100mL solution containing 2.5g BSA and 1g glucose which had been passed through a 0.22µm filter. Incubate solution (0.1mL) was transferred by sterile pipette to sterile glass tubes containing sterile basal medium (0.9mL) for decimal dilution. Relevant dilutions were transferred by pipette to plates and incubated for 48 hours at 37°C. Plates containing 3-30 independent colonies were scored. All dilutions were conducted in duplicate and the mean count from all plates within the scoring criteria was taken as the number of viable

aerotolerant bacteria. All enumerations were conducted within an hour of sample collection.

5.2.4 Monocultures of Actinomycetes

Two rumen incubates, RI-1 and RI-2, which had been shown to inhibit gastrin release in the *in vitro* tissue incubation system, were each inoculated on to a basal medium-agar plate and bacteria were grown aerobically for five days at 37°C. The predominant colony in each case was identified by Dr. Andrew Hudson of AgResearch Grasslands as a member of the Actinomycete family. A single colony was picked off each plate and grown for 10 days in sterilised basal medium at 37°C, and a subsample taken after five days.

5.2.5 Culture of *Streptococcus bovis*

A colony of *S. bovis* from a monoculture on a Brain-Heart-Infusion agar plate (donated by Dr. Keith Joblin, AgResearch Grasslands) was grown in 50mL of sterilised basal medium at 37°C for 10 days, and a subsample taken after five days.

5.2.6 *In vitro* tissue incubation test system

The effects of abomasal fluids, rumen fluids, abomasal incubates and rumen incubates on *in vitro* gastrin secretion were examined using the tissue incubation method described in Section 2.2.1. Before testing, incubates were diluted 10-fold with basal medium and the pH adjusted to 7.40±0.02. The osmolarity was measured (Section 2.2.1) and was always between 320-340 $mOsm.L^{-1}$. All test solutions were prepared no more than three hours before *in vitro* tissue incubations were conducted.

5.2.6.1 Exposure to antibiotics

To determine whether bacterial metabolism causes the inhibitory effects observed in the *in vitro* tissue preparation, antibiotics were added to the basal medium used in the tissue test. The effects of antibiotics in the incubation

medium for the tissue test on the *in vitro* tissue gastrin secretion response to rumen contents was tested on three occasions (RUM-1, -3 and -6), with 1mL.L^{-1} Penicillin/Streptomycin and 25mg.L^{-1} Kanamycin present in the test solution. One rumen fluid sample (RUM-1) was tested with basal medium containing 0.2g.L^{-1} neomycin. The effect on basal gastrin secretion of each medium containing antibiotics was also tested.

5.2.6.2 Acidification and alkalinisation

The effect of altering the pH of rumen fluids and rumen incubates on the *in vitro* tissue gastrin response they produce was tested. The pH of one aliquot of each of six rumen fluids (RUM-2, RUM-3, RUM-4, RUM-5, RUM-6, RUM-7) and four rumen incubates (RI-18 and RI-21, RI-22 and RI-23) was adjusted to pH 5.5, 5, 4.5, 4, 3.5, 3 or 2 with 1M HCl and the solutions kept at room temperature for 30 minutes. The pH was then adjusted back to 7.40 with 1M NaOH. The pH of two rumen fluid samples (RUM-6 and RUM-7) was adjusted to pH 8, 10, 12 and 13 with 1M NaOH and similarly incubated.

The effects of pH adjustment on the *in vitro* tissue gastrin response were compared to control solutions and the original, non pH-adjusted, rumen or rumen incubate solution.

5.2.6.3 Increased and decreased temperature

The effect of increasing the temperature to 45°C, 55°C, 70°C and 100°C for 30 minutes was tested on two rumen fluid samples (RUM-3 and -5). The effect of refrigerating (4°C) or freezing (-18°C) for one week prior to testing was examined on one rumen fluid sample (RUM-5). The effect of increasing the temperature to 45°C and 55°C for 30 minutes was tested on four incubate samples known to have *in vitro* inhibitory activity (RI-18, -21, -22, -23).

After being maintained at raised temperature, all test solutions were cooled to 4°C and diluted 10-fold in basal medium heated to 37°C before they were tested in the *in vitro* system.

After exposure to low temperature, solutions were brought to room temperature, diluted 10-fold, then warmed to 37°C and tested. Untreated rumen solutions were also tested, as was basal media treated in similar fashion to test solutions.

5.2.6.4 Filtration

Seven rumen fluids (RUM-8 to -13) and six rumen incubates (RI-3 to RI-8) were filtered through a N_2 pressurised filter system which passes solutions though a dual membrane, the first a prefilter (Millipore, AW0314250) and the second a 0.22μm hydrophilic filter (Millipore, GVWP142 50) to remove all bacteria. The bacteria and particulate matter retained by the filter and prefilter were resuspended in basal medium in the same volume as removed by filtration. Suspensions were tested for gastrin inhibitory activity. On three occasions, the prefilter filter was soaked in 50mL of basal medium for 30 minutes and the medium then tested for inhibitory activity.

5.2.6.5 Centrifugation

Samples were centrifuged at 19 500*g* for 30 minutes to remove particulate matter and at 49 500*g* for one hour to remove bacteria. Four rumen samples were centrifuged at 19 500*g* and tested on four separate occasions (RUM-14 to RI-17 a, b, c, d); thus samples c were frozen and thawed twice. The same four original samples were centrifuged at 49 500*g* and tested on three separate occasions (RUM-14 to RI-17 c, d). These solutions were frozen between experiments and centrifuged on the day of testing; thus samples b were frozen and thawed once, samples c twice and d three times.

Effects of centrifugation at 49 500*g* were tested on three abomasal incubates (AI-8, AI-9 and AI-10).

Effects on centrifugation at 19 500*g* were tested on four incubates twice (RI-24 to -27 b, c) and at 49 500*g* on four incubates three times (RI-24 to -27 b, c, d), and on a further three incubates once (RI-21 to -23).

5.2.7 Effect of rumen and abomasal incubates on gastrin and somatostatin RIA

The effect of a final concentration of 1% of three abomasal incubates (AI-3, AI-4, and AI-5) and four rumen incubates (RI-5, RI-6, RI7 and RI-8) *on* maximum radioligand binding (zero) and non-specific binding (blank) in the gastrin and somatostatin radioimmunoassays (Appendix 1 and 2 respectively) was tested. A 1% concentration was used as this is the maximum amount of

rumen fluid or rumen incubate that would be present in RIA tests during assessment of *in vitro* tissue tests.

5.2.8 Gastrin loss from standard solutions ("breakdown" activity)

A total of 12 rumen fluid samples (RUM-14 to -26), four abomasal fluid samples (ABO-1 to -4), three abomasal incubates (AI-8, AI-9 and AI-10) and 15 rumen incubates (RI-15 to -29) were incubated with gastrin standards to estimate the "breakdown" of gastrin during *in vitro* test incubations. The method is described in Section 4.2.4.1. Rumen fluid or rumen incubate solutions were added to 50pM, 100pM, 200pM and 400pM gastrin standard solutions (RUM-14 to -17 and RUM-18 to -23 and RI-15 to -20 RI-24 to -27 a, b, c), or 50pM, 100pM and 200pM gastrin standard solutions (RI-21 to -23 and RI-24 to -27d), or with four 100pM gastrin RIA standard solutions (ABO-1 to -4, RUM-26 to -26, RI-28, RI-29) such that rumen contents made up 10% (v/v) of the final solution. Control standard solutions had 10% (v/v) Veronal buffer added. Both test and control solutions were then incubated for 30 minutes at 37°C, then cooled to 4°C and incubated at that temperature for three hours, then stored at -18°C until assayed. The protocol was designed to mimic incubation incubations conducted in the *in vitro* test system (described in Section 2.2.1).

Details of testing were as follows:

I. Four rumen fluid samples (RUM-14 to -17) were tested four times over a period of 40 days. Samples were stored at -18°C between tests. Four rumen incubates (RI-24 to -27) were tested three times over 30 days and once again, two months later.

II. Six rumen incubates (RI-15 to -20) were tested after incubation for periods of 40 or 64 hours.

III. Four rumen incubates were tested after acidification of sample (samples and method described in 5.2.6.2).

IV. Four rumen fluids (RUM-14 to -17) and four rumen incubates (RI-24 to -27) were centrifuged at 19 500*g* for 30 minutes. Seven rumen samples and six rumen incubates were centrifuged at 49 500*g* for 60 minutes.

V. Four abomasal fluids (ABO-1 to -4) of low pH were tested untreated, after centrifugation at 19 500*g* for 30 minutes and at 49 500*g* for 60 minutes.

VI. Three abomasal incubates (AI-8, AI-9 and AI-10) were tested untreated and after centrifigation at 49 500*g* for 30 minutes.

VII. Three rumen fluids (RUM-24 to -26) and one rumen incubate (RI-29) were added to basal medium containing a final concentration of 10, 1 and 0.1mg.mL^{-1} of a general bacterial protease inhibitor mixture (P8465, Sigma Chemical Co., USA) and tested in the *in vitro* system, and with standards with the same concentrations of protease inhibitor added.

5.2.8.1 Calculations

The gastrin breakdown or "reduction ratio" was calculated by dividing the gastrin concentration in the standard/test mixture by the gastrin concentrations in the control standard solution. The mean±SEM was calculated for the four standards. Results were converted to percentage values for Tables and expressed as "breakdown."

To determine whether breakdown could wholly account for gastrin reduction in tissue incubations, gastrin concentrations expected after allowing for breakdown and control decline in gastrin secretion were calculated and compared with observed results. The gastrin concentration expected in each of the B solutions was calculated from the concentration of gastrin in the A solution, the observed decline in gastrin secretion from control plates and the breakdown of standards. Thus, the A value for each tissue piece was multiplied by the B/A ratio from control plates (forming B_1) and then by the reduction ratio from standards (forming B_2). The B_2 results were then compared with the observed result (B actual) in tissue plate using a paired t test in SPSS version 9.0 with significance level set at $p<0.01$.

5.2.9 Statistical analyses

All data from *in vitro* tissue incubations are presented as mean±SEM for each plate (eight pieces of tissue) unless otherwise indicated; "n" is the number of replicate experiments. The effects of test solutions were determined using UNIANOVA in SPSS version 9.0. All data were examined for normality using Shapiro-Wilks test and for equality of variance using Levene's Homogeneity of Variance test. *Post hoc* comparisons of data groups when variances were equal were conducted using Tukey's HSD test for comparisons of all groups, or Dunnett's test for comparisons with control alone. When variances were unequal, Tamhane's T2 *post hoc* test was used. When the data were not normally distributed, Kruskal Wallis analysis in Graphpad Prism version 2.01, with Dunn's *post hoc* test were used. Differences between breakdown percentages were calculated using one-way ANOVA with the same data considerations as used for UNIANOVA. Paired t-tests were conducted in SPSS version 9.0, with the same data consideration as used for UNIANOVA tests.

5.3 Results

For the results presented below, gastrin inhibitory activity is described in three ways. Tissue gastrin inhibitory activity refers to the total *in vitro* gastrin response to test solutions. The "breakdown" effect refers to the component of activity which could be explained by "breakdown" activity as estimated by incubation of standards with test solutions. Inhibition of secretion and secretion inhibitor refers to that component of the gastrin inhibitory activity which cannot be explained by "breakdown" activity.

5.3.1 Effects of abomasal and rumen incubates on RIA's

Neither rumen (n=4) nor abomasal contents (n=3) at a concentration of 1% affected radioligand or non-specific gastrin binding in the gastrin RIA, even when an inhibitor of tissue gastrin secretion was present in solutions.

Before incubation and until tissue gastrin inhibitory activity was observed in solutions, incubates had no effect on radioligand and non-specific binding in

Sample	"Breakdown"	20K "breakdown"	50K "breakdown"
ABO-1	12±20%	-5±2%	-2±11%
ABO-2	2±2%	11±18%	-10±4%
ABO-3	-4±6%	-4±9%	-19±4%
ABO-4	8±10%	-4±3%	2±12%

Table 5.1. Estimation of gastrin "breakdown" activity in 4 abomasal samples from unparasitised animals, untreated, centrifuged at 19 500*g* (20K), and at 49 500*g* (50K). Breakdown activity is expressed as mean±SEM. None of the abomasal samples affected *in vitro* gastrin secretion in the tissue assay. Breakdown activity is expressed as percentage, derived from a ratio of gastrin standard solution incubated with abomasal sample divided by untreated incubated standard solution.

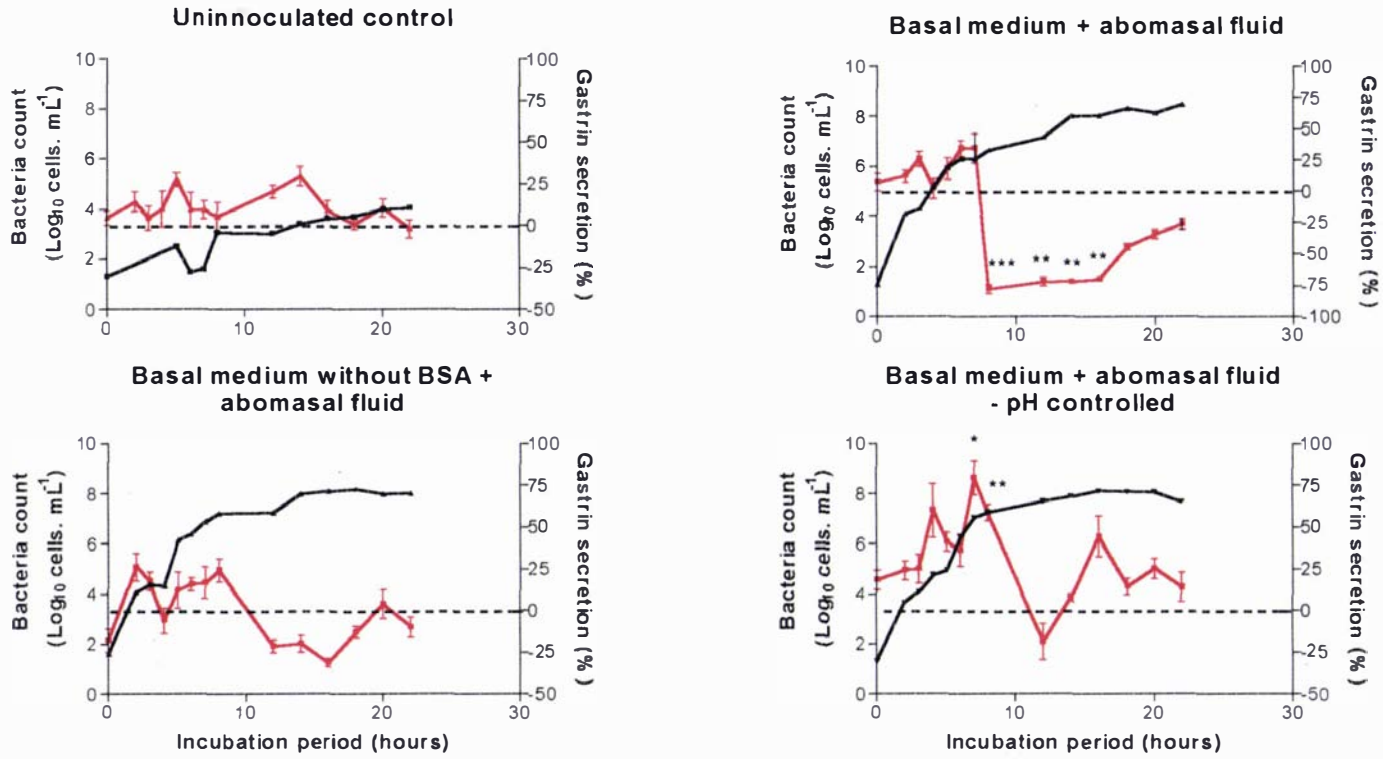

Figure 5.1. *In vitro* gastrin response (⟵) to and bacterial numbers (⟵) in incubates of abomasal contents in normal basal medium, basal medium without BSA, and basal medium where pH is maintained between 7.20-7.40 inoculated with abomasal fluid. Gastrin response is expressed as mean±SEM, with the dashed horizontal line showing control gastrin secretion. Basal medium consists of Hank's balanced salt solution, with 0.25% BSA, 0.1% glucose, 10mM HEPES, 0.35g.L^{-1} $NaCO_3$, and is at pH7.40 at start of incubation. Abomasal fluid made up 1% of the final incubate volume in all incubate solutions except uninnoculated control, *=p<0.05, **=p<0.01, ***=p<0.001 gastrin response significantly different from control.

the somatostatin RIA. When incubate samples contained tissue gastrin, inhibitory activity markedly affected antiserum binding and non-specific binding in the somatostatin assay, producing large values for both. Thus, the effects of gastrin inhibitory activity of incubates on somatostatin secretion could not be studied.

5.3.2 Inhibitory activity in abomasal samples and abomasal incubates

5.3.2.1 Abomasal fluids

Abomasal fluids (ABO1-4) which had a pH between 2.4 and 2.8 did not have any effect on gastrin secretion ($p>0.05$) and did not exhibit any gastrin "breakdown" activity (Table 5.1), nor did these samples have any effect on gastrin secreted in the *in vitro* tissue assay. Results did not differ when samples were centrifuged at 19 500*g* and 49 500*g*.

5.3.2.2 Abomasal incubates

Nine of 10 abomasal samples incubated in basal medium produced tissue gastrin inhibitory activity. Prior to incubation the basal medium containing abomasal solution had no effect on basal gastrin secretion ($p>0.05$), but after incubation for 12 to 96 hours, solutions contained gastrin inhibitory activity. None of these samples were tested for gastrin "breakdown" activity.

When bacterial numbers were estimated (AI-1 and AI-2), the appearance of inhibitory activity coincided with the highest numbers of bacteria, which was in the stationary growth phase (Figure 5.1). All incubates, both rumen and abomasal, containing inhibitory activity were turbid and, in most cases, the pH of the solution was markedly reduced.

Omission of BSA or maintaining the pH of the medium close to that of uninnoculated basal medium reduced or abolished the production of the inhibitory action in AI-1 incubates. Similar results were noted in the replicate study (AI-2), where inhibitory activity was present in the sample incubated in standard basal medium after 12 hours of incubation, in the control solution after 24 hours, but only after 48 hours in the pH-adjusted solutions and solutions

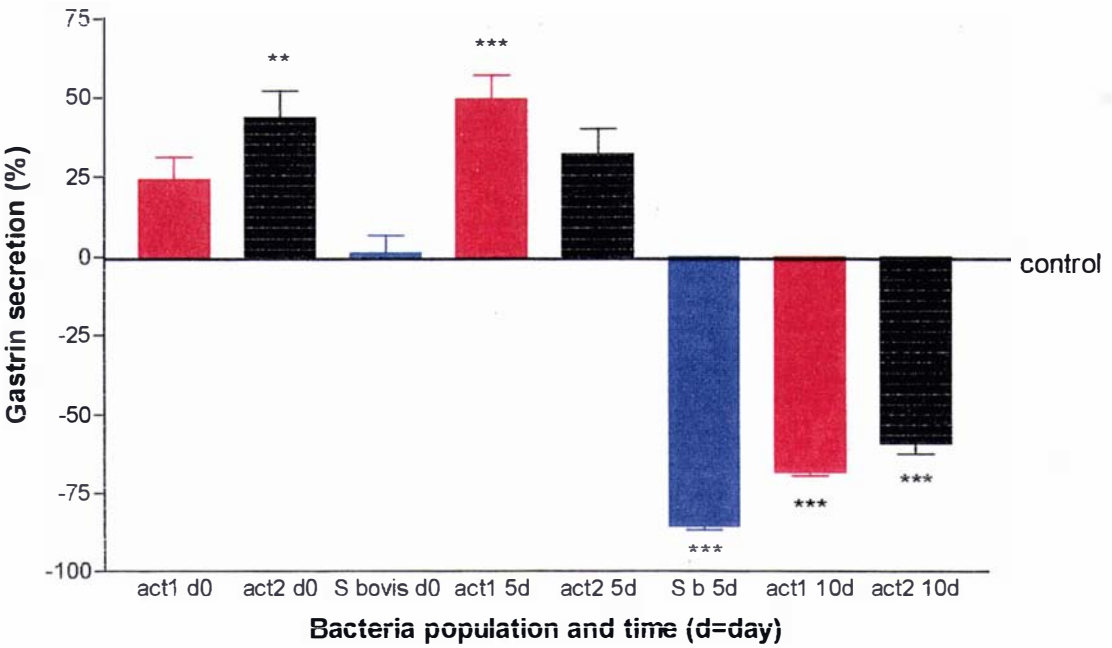

Figure 5.2. Effect of two Actinomycete (and) and one *S. bovis* monoculture ( ) grown for 0, 5 and 10 days on *in vitro* gastrin secretion. Gastrin response is expressed as mean±SEM **=p<0.01, ***=p<0.001 gastrin secretion different from control.

where BSA was omitted. In AI-3, inhibitory activity was present in all solutions after 96 hours, but in none of the solutions from earlier time points. In AI-4, and AI-5, activity was present in sample incubated in standard basal medium after 24 hours in glucose-negative medium after 48 hours, but never present in solutions where BSA was omitted. AI-6 produced inhibitory activity after 24 hours of incubation, but AI-7 never produced inhibitory activity.

Tissue gastrin inhibitory activity was observed in AI-8, AI-9 and AI-10, and was accompanied by some "breakdown" activity in AI-9, but this did not account for all of the tissue gastrin inhibitory activity (tissue effect -92±1%, "breakdown" effect -31±5%, results significantly different $p<0.001$). There was little to no "breakdown" effect in AI-8 (-6±4%) or in AI10 (-10±3%). Centrifugation at 49 500*g* removed the tissue gastrin inhibitory activity in AI-9 (from -92±1% to -10±10%, $p<0.001$) and AI-10 (-87±2% to +23±10%, $p<0.01$) and reduced the "breakdown" activity in AI-9 (from -31±5 to -10±5%, $p<0.05$).

In two cases, AI and RI samples were obtained from the same animal, AI-4 with RI-1 and AI-5 with RI-2. AI-4 and RI-1 both produced inhibitory activity in normal medium after 24 hours and in medium without glucose in 48 hours, while RI-2 never produced inhibitory activity.

5.3.2.3 Inhibitory activity in monocultures

Both Actinomycete and *S. bovis* monoculture incubates produced gastrin inhibitory activity (Figure 5.2). Activity appeared first in *S. bovis* solutions, after five days of incubation. For both Actinomycete and *S. bovis* cultures, appearance of activity coincided with a reduction of the medium pH and increased turbidity of the solution. Gastrin "breakdown" activity was not assessed in these samples.

5.3.3 Inhibitory activity in rumen fluid and rumen incubates

5.3.3.1 Rumen fluid

All rumen fluid samples from lucerne and pasture fed animals contained tissue gastrin inhibitory activity. All rumen samples were taken from Sheep 1L to 4L, AI1 to AI6 and C1 to C4, each on three occasions. All contained tissue

Sample	Tissue response	Average Breakdown	Average A value	Average B_1	Average B_2	B actual	Statistic
RUM-18	-70±4%	-36±4%	215±41	189±36	121±22	52±9	ns
RUM-19	-81±1%	-36±7%	234±29	205±25	132±16	36±2	p<0.001
RUM-20	-80±2%	-37±4%	177±21	156±19	98±12	30±1	p<0.001
RUM-21	-76±1%	-33±9%	174±25	153±21	103±15	34±2	p<0.01
RUM-22	-77±1%	-40±5%	210±25	185±22	111±13	41±4	p<0.01
RUM-23	-74±2%	-39±3%	227±23	200±20	122±12	49±5	p<0.001

Table 5.2. Model calculations of the predicted gastrin concentration in the presence of test solution allowing for gastrin breakdown. All values are presented as mean±SEM. The gastrin concentration expected in each of the B solutions was calculated from the concentration of gastrin in the A solution, the observed decline in gastrin secretion from control plates and the breakdown of standards. Thus, the A value for each tissue piece was multiplied by the B/A ratio from control plates (forming B_1) and then by the reduction ratio from standards (forming B_2). The B_2 results were then compared with the observed result (B actual) in tissue plate using a paired t test in SPSS version 9.0 with significance level set at $p<0.01$. For comparison, the tissue response and reduction in standards (average breakdown), both expressed as a percentage are included.

Solution	Dilution	Generation time	Tissue response	Maximal Tissue response	Time of Maximal response
RI-3	1/500	20 hours	-96±1%	-96±1%	20 hours
RI-4	1/500	20 hours	-93±1%	-94±1%	40 hours
RI-5	1/500	20 hours	-85±8%	-93±1%	40 hours
RI-6	1/500	20 hours	-91±1%	-94±1%	40 hours
RI-7	1/500	20 hours	-92±2%	-93±1%	26 hours
RI-8	1/500	20 hours	-94±1%	-94±1%	20 hours
RI-9	1/500	14 hours	-91±1%	-91±1%	14 hours
RI-10	1/500	14 hours	-81±1%	-91±1%	70 hours
RI-11	1/500	14 hours	-94±1%	-94±1%	14 hours
RI-12	1/500	14 hours	-91±1%	-92±1%	48 hours
RI-13	1/500	14 hours	-85±2%	-93±1%	48 hours
RI-14	1/500	14 hours	-92±2%	-96±1%	48 hours
RI-15	1/500	12 hours	-88±3%	-90±2%	64 hours
RI-16	1/500	12 hours	-95±1%	-95±1%	12 hours
RI-17	1/500	12 hours	-93±1%	-93±1%	12 hours
RI-18	1/500	12 hours	-95±1%	-95±1%	12 hours
RI-19	1/500	12 hours	-95±1%	-95±1%	12 hours
RI-20	1/500	12 hours	-93±1%	-94±1%	80 hours
RI-21	1/500	24 hours	-57±6%	-57±6%	24 hours
RI-22	1/500	24 hours	-47±5%	-50±3%	48 hours
RI-23	1/500	24 hours	-31±7%	-58±6%	48 hours

Table 5.3. Production of tissue gastrin inhibitory activity by 20 rumen contents diluted in basal medium and incubated for periods of up to 96 hours. The table shows the dilution level of rumen contents to basal media (dilution), the first time that a significant *in vitro* gastrin inhibitory effect was noted in solution (generation time), the percentage inhibitory effect (tissue response), what the maximal inhibitory effect (maximal tissue response) was and at what time it occurred.

Sample	"Breakdown"	Tissue response
RI-15a	-23±6%*	-49±3%*
RI-15b	4±7%**	-65±7%
RI-16b	-19±2%**	-85±2%
RI-17b	-26±4%**	-82±2%
RI-18a	-28±5%**	-72±2%
RI-18b	-32±3%*	-88±6%
RI-19a	-22±5%**	-58±3%
RI-19b	-16±3%**	-93±1%
RI-20a	-64±1%	-60±6%
RI-20b	-83±2%	-89±1%

Table 5.4A Comparison between gastrin "breakdown" and tissue gastrin inhibitory activity in six rumen incubate samples of which four were tested after 40 hours (a), and all six tested after 64 hours (b) of incubation. All results are expressed as mean±SEM. For each sample, the tissue gastrin inhibitory activity (tissue response) and breakdown are shown, *= $p<0.01$, **= $p<0.001$ B_2 values, an estimate of the breakdown effect, are different from the observed values after incubation with tissue *in vitro.*

Sample	"Breakdown"	Tissue response
RI-24a	-52±1%**	-94±1%
RI-24b	-81±1%*	-90±1%
RI-24c	-94±2%*	-87±2%
RI-24d	-30±6%	-47±7%
RI-25a	-21±1%**	-94±1%
RI-25b	-80±2%*	-89±1%
RI-25c	-90±3%	-92±2%
RI-25d	-37±5%*	-85±2%
RI-26a	-42±3%**	-94±1%
RI-26b	-70±1%**	-90±1%
RI-26c	-91±4%	-92±2%
RI-26d	-43±10%*	-84±2%
RI-27a	-36±4%**	-93±1%
RI-27b	-73±5%*	-88±1%
RI-27c	-93±1%	-91±1%
RI-27d	-41±3%*	-89±3%

Table 5.4B Comparison between gastrin "breakdown" and tissue gastrin inhibitory activity in four rumen incubate samples each tested four times (a-d). All results are expressed as mean±SEM. For each sample, the tissue gastrin inhibitory activity (tissue response) and breakdown are shown, *= $p<0.01$, **= $p<0.001$ B_2 values, an estimate of the breakdown effect, are different from the observed values after incubation with tissue *in vitro.*

gastrin inhibitory activity (between -73±4% to -94±1%). The degree of tissue gastrin inhibitory activity was not affected by sampling day, whether animals were parasitised with adult or larval *O. circumcincta*, nor did activity vary between animals ($p>0.05$).

All rumen samples when first tested contained tissue gastrin inhibitory activity. In seven of the 13 cases where tested, gastrin "breakdown" and tissue gastrin inhibitory activity were not different ($p>0.05$). In such cases gastrin "breakdown" could fully explain tissue gastrin inhibitory activity.

Calculations of gastrin "breakdown" by rumen solutions are shown in Table 5.2. For RUM-19 to RUM-23, not all tissue gastrin inhibitory activity could be explained by gastrin "breakdown," as there was a significant difference between the B_2 value and the observed B value. In general, for RUM-19 to RUM-23, of the 70-80% tissue gastrin inhibitory activity caused by rumen contents, 33-40% (nearly half) could be accounted for by gastrin "breakdown." Although the tissue gastrin inhibitory activity and "breakdown" percentage results for RUM-18 do not appear to be different from those of RUM-19 to RUM-23, there is greater variation in both the B_2 and the actual B values, resulting in no statistical difference between these values. In other rumen samples (RUM-14 to RUM-17 and RUM-24 to RUM-26), all the inhibitory activity could be accounted for by gastrin "breakdown" activity.

5.3.3.2 Rumen incubates

Incubation of 22 of 23 rumen fluid samples in basal medium produced gastrin inhibitory effects (Table 5.3). Prior to incubation, the basal medium containing abomasal solution had no effect on basal gastrin secretion ($p>0.05$). In general, once activity was observed it was maintained for the duration of the incubation. Appearance of inhibitory activity again coincided on most occasions with medium becoming turbid and more acidic, sometimes as low as pH6. Some samples (RI-15 to RI-20) did develop activity before pH decreased below pH7, but were turbid when tissue gastrin inhibitory activity first appeared.

Rumen incubates contained "breakdown" activity in 11 out of 15 cases. In two incubates, "breakdown" could account for all of the tissue gastrin inhibitory activity in the first test of the sample (RI-20 Table 5.4A and RI-29,

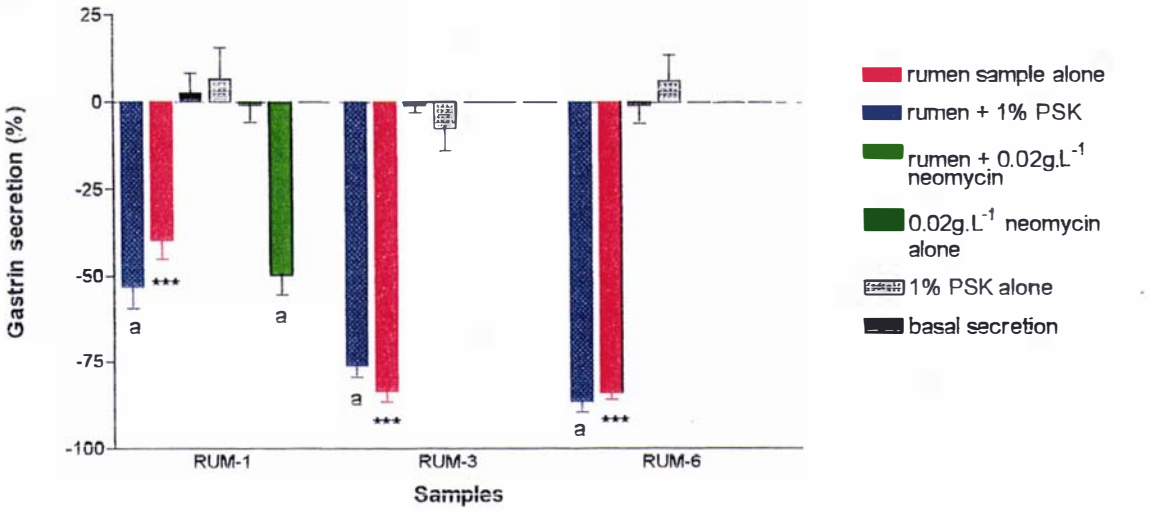

Figure 5.3. *In vitro* gastrin response to rumen contents in the presence of 1% Penicillin-Streptomycin-Kanamycin mixture or 0.2g L^{-1} neomycin. Results from three experiments, each with a different rumen fluid solution are shown, along with the effects of antibiotics on basal gastrin secretion. Gastrin response is expressed as mean±SEM ***=p<0.001, gastrin secretion different from control, a=p<0.001, gastrin secretion different from control but not (p>0.05) different from effects of rumen solution alone.

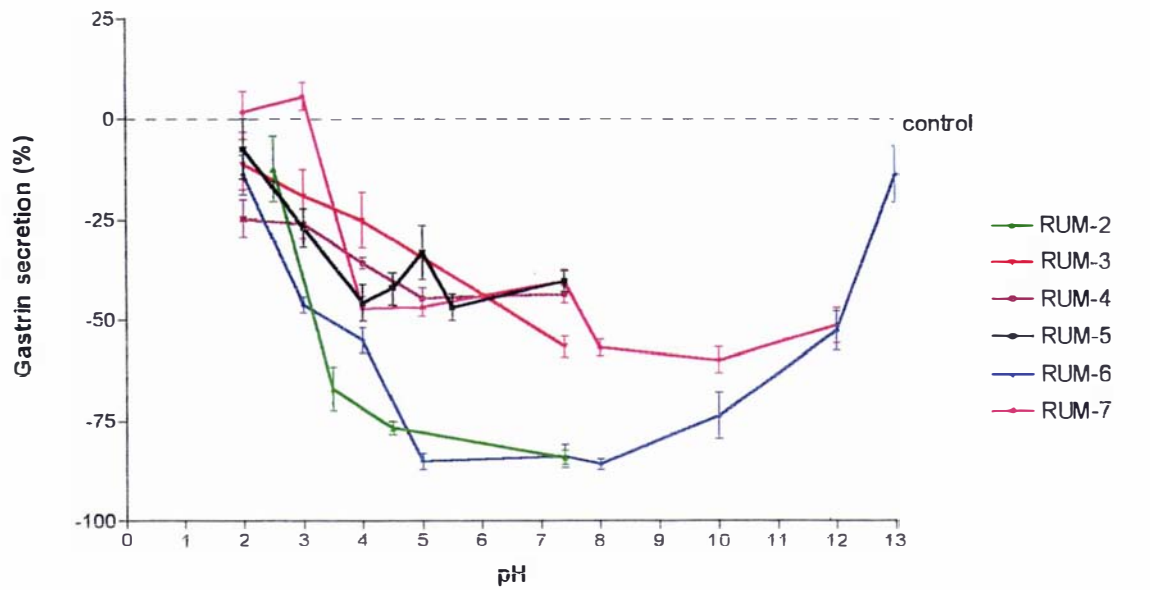

Figure 5.4. *In vitro* gastrin response to six rumen fluids exposed to pH2-14 at room temperature for 30 minutes prior to test incubation. Gastrin response is expressed as mean±SEM. Statistics for the data are shown in Table 5.5.

Treat pH	RUM-2 Resp	RUM-2 Stats	RUM-3 Resp	RUM-3 Stats	RUM-4 Resp	RUM-4 Stats	RUM-5 Resp	RUM-5 Stats	RUM-6 Resp	RUM-6 Stats	RUM-7 Resp	RUM-7 Stats
2			-4±5	b***	-25±4	a***b**	-4±6	b*	-14±5	b***	+2±5	b***
2.5	-13±6	b***										
3			-23±4	a*b***	-26±3	a***b**	-27±4	-	-46±1	a***b***	+6±3	b***
3.5	-72±3	a***										
4			-22±6	b***	-36±1	a***	-40±4	a***	-55±3	a***b**	-47±3	a***
4.5	-76±3	a***					-46±3	a***				
5					-45±2	a***	-40±4	a***	-85±2	a***	-47±2	a***
5.5							-47±4	a***				
8									-85±2	a***	-57±2	a***b*
10									-74±6	a***	-60±3	a***b*
12									-53±5	a***b*	-54±3	a***
13									-14±6	b**		

Table 5.5. Statistics for *in vitro* gastrin response to six rumen fluid samples exposed to pH2-14 at room temperature for 30 minutes prior to test incubation. Treat pH = the pH rumen solution was altered to prior to testing, Resp= the *in vitro* tissue gastrin response to the treated rumen sample, shown as mean±SEM, Stats = the statistical differences of the response to untreated samples and basal gastrin secretion. For statistics a = gastrin secretion in response to solution is different from basal (control) levels, b = gastrin response to treated sample is different from the gastrin response to the untreated rumen sample, *= $p<0.05$, ** = $p<0.01$, *** = $p<0.001$.

Table 5.4B). In the other 13 incubates tissue gastrin inhibitory activity could not be explained by "breakdown" effects.

Of the six samples described in Table 5.4A, five (RI-15 to RI-19) showed very little gastrin "breakdown" activity (19-32%) despite causing 50-93% inhibition of gastrin secretion *in vitro.* Incubation for an extra 24 hours (40 versus 64 hours) did not cause significant changes in the reduction ratios.

Freezing and thawing rumen incubates caused significant increases ($p<0.05$) in the amount of breakdown activity in the four rumen incubates tested four times, such that "breakdown" activity was no longer significantly different from inhibitory activity (Table 5.4B). This was the case for RI-24, RI-25 and RI-27 where breakdown in 'a' was less than in 'b' and 'c' and in R-26 where 'a' was different to 'c.' This breakdown activity was markedly reduced ($p<0.05$) from in the last test (d) of RI-24 to RI-27 when compared with 'c,' though in three of the four cases (RI-25, RI-26 and RI-27), this was not accompanied by a reduction in inhibitory effect in tissue tests (Table 5.4B).

5.3.3.3 Properties of activity in rumen fluids and rumen incubates

5.3.3.3.1 Antibiotic treatment

Presence of 1% Penicillin-Streptomycin-Kanamycin mix or Neomycin in the *in vitro* tissue test incubation medium did not affect ($p>0.05$) basal gastrin secretion, or the tissue gastrin inhibitory activity of rumen contents (Figure 5.3). These antibiotics were effective in inhibiting bacterial growth and production of inhibitory activity in two rumen (RI-1 and RI-2) and two abomasal (AI-4 and AI-5) incubations.

5.3.3.3.2 Altered pH

The effects of decreasing or increasing the pH of rumen fluid on its tissue gastrin inhibitory activity is shown in Figure 5.4, with details of responses shown in Table 5.5. Altering the pH of basal medium then returning it to pH 7.40 and then diluting 10-fold in untreated basal medium for testing did not affect gastrin secretion ($p>0.05$). With RUM-3, gastrin secretion was also reduced when tissue incubation medium contained control basal medium solutions which were decreased to pH2 (-18±1%), pH3 (-10±2%), pH4 (-9±4%)

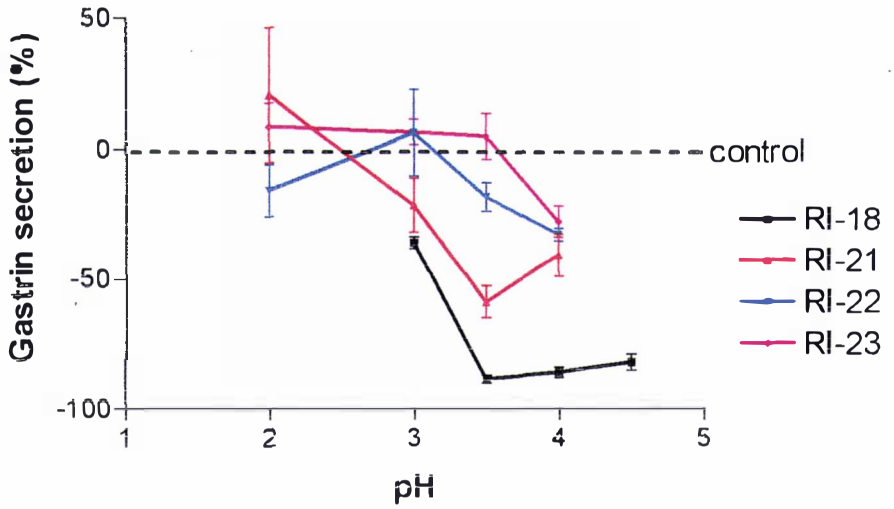

Figure 5.5. *In vitro* gastrin response to four rumen incubate solutions exposed to pH2-4.5at room temperature for 30 minutes prior to test incubation. Gastrin response is expressed as mean±SEM. Statistics for data are shown in Table 5.6.

Sample number	pH2		pH3		pH3.5		pH4	
	Resp	sig	Resp	sig	Resp	sig	Resp	sig
RI-18	-	-	-36±2%	ab	-89±1%	a	-82±1%	ab
RI-21	+20±26%	-	+6±32%	-	-59±6%	a	-31±14%	a
RI-22	-14±10%	-	+6±16%	b	-18±5%	-	-33±2%	a
RI-23	+8±9%	b	+7±5%	b	+4±8%	b	-28±6%	a

Table 5.6. Statistics for the *in vitro* gastrin response to four rumen incubate samples exposed to pH2-4 at room temperature for 30 minutes prior to test incubation. Resp = the gastrin response to the treated rumen sample, shown as mean±SEM. Sig = the statistical differences of the response to untreated samples and basal gastrin secretion. For statistics a = gastrin secretion in response to solution is different from basal (control) levels, b = gastrin response to treated sample is different from the gastrin response to the untreated rumen sample.

Sample	UNTREATED		pH2		pH3		pH3.5		pH4	
	Tissue	b/d	Tissue	b/d	Tissue	b/d	Tissue	b/d	Tissue	b/d
RI-27d	-89±3%	-41±3%*	-	-	-35±2%	-10±5%	-88±1%	3±6%**	-86±1%	-12±4**
RI-21	-55±4%	10±15%**	+20±20%	12±23%	-22±9%	8±13%	-59±6%	7±2%**	-41±8%	12±7%*
RI-22	-33±4%	5±13%**	-16±9%	10±14%	+6±15%	11±7%	-19±4%	8±8%**	-33±2%	8±7%*
RI-23	-39±4%	4±4%**	+8±8%	1±15%	+6±5%	-6±7%	+5±8%	11±16%**	-28±4%	4±12%

Table 5.7. Comparison between gastrin "breakdown" and tissue gastrin inhibitory activity in four rumen incubates untreated and exposed to pH2-4 for 30 minutes prior to *in vitro* testing. All results are expressed as mean±SEM. For each sample, the tissue gastrin inhibitory activity (tissue) and breakdown (b/d) effects are shown, *= $p<0.01$, **= $p<0.001$ B_2 values, an estimate of the breakdown effect, are different from the observed values after incubation with tissue *in vitro*.

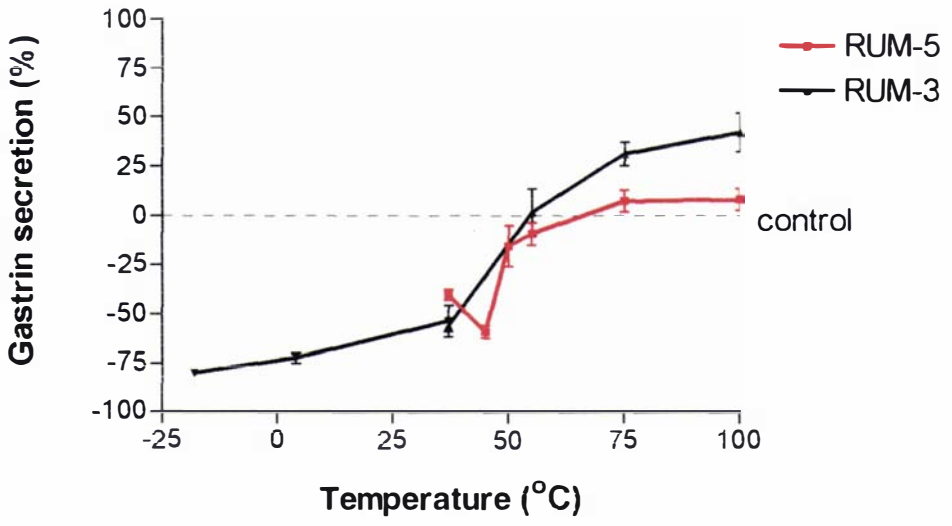

Figure 5.6. *In vitro* gastrin response to two rumen fluids incubated at -18 to 100°C prior to test incubation. Gastrin response is expressed as mean±SEM. Statistics for data are shown in table 5.4.

Temp	RUM-3		RUM-5	
°C	Response	Statistics	Response	Statistics
-18	-80±4%	a***b***		
4	-54±8%	a***		
45	-59±4%	a***		
50	-12±10%	b***		
55	-13±6%	b***	+1±12%	b*
75	+8±8%	b***	+31±6%	a**b***
100	+8±5%	b***	+42±10%	a*b***

Table 5.8. Statistics for the *in vitro* gastrin response to the rumen fluid samples incubated at -18 to 100°C prior to test incubation. Response = the tissue gastrin response to the treated rumen sample, shown as mean±SEM. Statistics = the statistical differences of the response to untreated samples and basal gastrin secretion. For statistics a = gastrin secretion in response to solution is different from basal (control) levels, b = gastrin response to treated sample is different from the gastrin response to the untreated rumen sample, *= $p<0.05$, ** = $p<0.01$, *** = $p<0.001$.

and pH4.5 (-7±4%). This may have been due to the increased osmolarity of these tissue incubation solutions resulting from pH adjustments (415, 390, 360 and 370mOsm.L^{-1} for pH2, 3, 4 and 4.5 controls respectively). Tissue incubation solutions containing rumen contents, even pH altered rumen contents, in this and all other experiments had osmolarity between 315 and 336mOsm.L^{-1}.

While in general, decreased pH had reduced the amount of inhibitory activity, the precise sensitivity of tissue gastrin inhibitory activity in rumen fluid was unclear. *Post hoc* comparisons between different pH treatments revealed varied pH sensitivity of tissue gastrin inhibitory activity between samples. In summary lowering pH to 3 removed tissue gastrin inhibitory activity in three out of four tests. Tissue gastrin inhibitory activity after reduction of rumen fluid pH to 4 was variable, with significant removal in two of the six rumen fluids. Thus, there is near maximal removal at pH2 and maximal survival at pH5, but variable results between pH3 and 4.5.

Sensitivity to pH of tissue gastrin inhibitory activity in rumen incubate samples is illustrated in Figure 5.5 and described in Table 5.6. Lowering pH to 2 and 3 reduced or removed tissue gastrin inhibitory. In one of the four rumen incubate samples, reduction of pH to 3.5 also removed tissue gastrin inhibitory activity, but activity was intact at pH4 in all samples.

Table 5.7 shows the effect of lowering pH on the breakdown effect of rumen incubates. In only one case where this was tested was there gastrin "breakdown" in the original solution (RI-27d). In this example, lowering pH to 3 and 3.5 removed "breakdown" activity ($p<0.05$) which was also reduced at pH4 ($p<0.05$, less than original). In contrast, the gastrin secretion inhibitor was intact at pH3.5 and pH4 in RI-27d as in RI-21 and RI-23 ($p>0.05$, not different from original). Secretion inhibitor was destroyed at pH2 and pH3 ($p<0.05$, less than the original) in all samples, and was also removed at pH3.5 for RI-23 ($p<0.05$ less than original).

5.3.3.3.3 Temperature

Figure 5.6 and Table 5.8 illustrate the effects of temperature on rumen tissue gastrin inhibitory activity. In general, increasing temperature to levels above 55°C resulted in near complete degradation of tissue gastrin inhibitory

Sample number	Untreated		45°C		55°C	
	Resp	Stats	%	sig	%	sig
RI-18	-89±1%	a***	-85±1%	a***	-49±10%	a**b***
RI-21	-87±1%	a***	-	-	-91±1%	a***
RI-22	-88±1%	a***	-92±1%	a***	-90±1%	a***
RI-23	-90±1%	a***	-92±1%	a***	-82±1%	a***

Table 5.9. Statistics for the *in vitro* gastrin response to four rumen incubate samples exposed to pH2-4 at room temperature for 30 minutes prior to test incubation. Resp = the gastrin response to the treated rumen sample, shown as mean±SEM, Stats = the statistical differences of the response to untreated samples and basal gastrin secretion. For statistics a = gastrin secretion in response to solution is different from basal (control) levels, b = gastrin response to treated sample is different from the gastrin response to the untreated rumen sample.

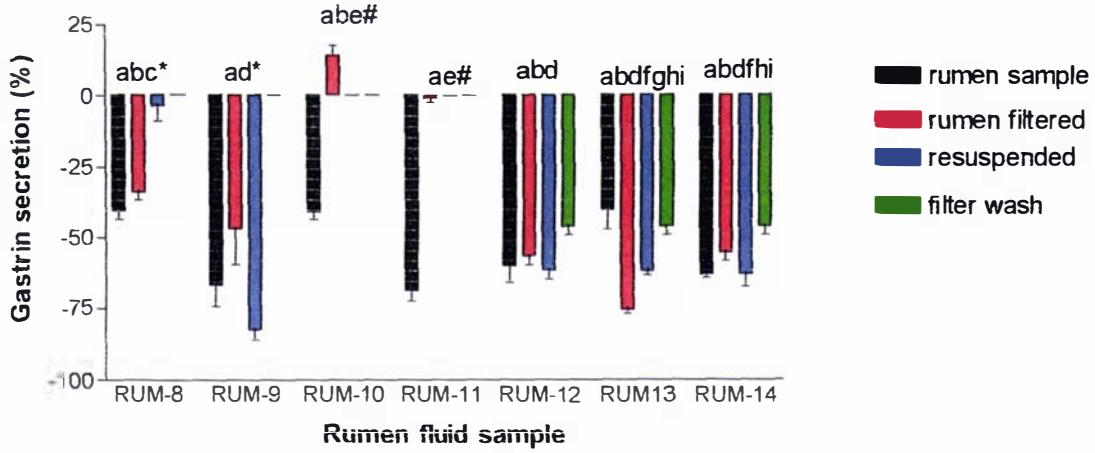

Figure 5.7. *In vitro* gastrin response to seven rumen fluids after passage through a 0.22μm filter. Gastrin response is expressed as mean±SEM. Shown is the gastrin response to the original rumen solution, filtered solution, material removed by filter resuspened in basal medium, material washed off filter in basal medium; a=original solution different from control, b=filtered rumen different from control, c=resupended different to rumen, *=no filter wash, d=resuspended different to control, e=filtered different from rumen, #=no resuspended or filter wash, f=filter wash different to control, g=filtered different from resuspended, h=filtered different from filter wash, i=resuspended solution different from filter wash.

Treatment	UNTREATED		20K CENT	
Sample	tissue response	"breakdown"	tissue response	"breakdown"
RUM-14a	-71±4%	-47±6%	-83±2%	-29±2%*
RUM-14b	-68±1%	-35±3%**	-70±2%	-42±4%**
RUM-14c	-77±1%	-80±5%	-78±1%	-79±2%
RUM-14d	-76±2%	-89±4%**	-69±2%	-93±1%**
RUM-15a	-52±6%	-49±4%	-59±3%	-37±2%*
RUM-15b	-59±4%	43±5%	-62±2%	-37±5%**
RUM-15c	-79±1%	-77±6%	-76±1%	-77±3%
RUM-15d	-73±2%	-86±5%**	-88±1%	-94±3%**
RUM-16a	-78±4%	-52±5%*	-53±6%	-40±4%
RUM-16b	-65±2%	-42±4%*	-70±1%	-42±5%**
RUM-16c	-63±2%	-76±6%*	-67±2%	-68±3%
RUM-16d	-70±3%	-93±4%**	-90±1%	-92±3%
RUM-17a	-72±8%	-53±9%	-61±2%	-54±3%
RUM-17c	-72±2%	-73±7%	-64±2%	-79±1%
RUM-17d	-73±3%	-90±4%**	-81±1%	-92±2%**

Table 5.10A. Comparison between gastrin "breakdown" and tissue gastrin inhibitory activity in four rumen fluids tested four times, untreated and after centrifugation at 19 500*g* (20K cent). All results are expressed as mean±SEM. For each sample, the tissue gastrin inhibitory activity (tissue response) and breakdown are shown, * = $p<0.01$, **= $p<0.001$, B_2 values, an estimate of the breakdown effect, are different from the observed values after incubation with tissue *in vitro*.

Treatment	UNTREATED		50K CENT	
Sample	tissue response	"breakdown"	tissue response	"breakdown"
RUM-14d	-76±2%	-89±4%**	-67±2%	-90±4%**
RUM-15d	-73±2%	-86±5%**	-59±3%	-93±3%**
RUM-16d	-70±3%	-93±4%**	-73±2%	-92±2%*
RUM-17d	-73±3%	-90±4%**	-75±2%	-92±2%**
RUM-24	-81±4%	-81±3%	-78±3%	-63±5%
RUM-25	-81±3%	-76±5%	-72±4%	-53±6%
RUM-26	-86±2%	-83±3%	-67±2%[a]	-75±4%

Table 5.10B. Comparison between gastrin "breakdown" and tissue gastrin inhibitory activity in seven rumen fluids, untreated and after centrifugation at 49 500*g* (50K cent). All results are expressed as mean±SEM. For each sample, the tissue gastrin inhibitory activity (tissue response) and breakdown are shown, **= $p<0.001$ B_2 values, an estimate of the breakdown effect, are different from the observed values after incubation with tissue *in vitro*, [a]=$p<0.05$ centrifugation reduced tissue gastrin inhibitory activity.

activity in rumen fluid, while freezing solutions in a standard -18°C freezer for seven days increased inhibitory activity.

The tissue gastrin inhibitory activity in incubate samples was not sensitive to temperatures of 45 or 55°C (Table 5.9).

5.3.3.3.4 Filtration and Centrifugation

Filtration of rumen contents removed tissue gastrin inhibitory activity in rumen solutions in two (RUM-10 and RUM-11) of the seven solutions tested (Figure 5.7). In four of the five samples where it was tested, material retained by the filter papers contained inhibitory activity, as did the filter soaked in basal medium (the filter wash). Filtration did not reduce tissue gastrin inhibitory activity of the solution in four of the five occasions (RUM-8, RUM-9, RUM-12 and RUM-14) when activity appeared in the filtrate, and on the other occasion (RUM-13) filtration increased tissue gastrin inhibitory activity.

Filtration removed tissue gastrin inhibitory activity (RI-6 and RI-7, $p>0.05$, filtered sample versus control) in two of the six rumen incubate samples tested, and reduced activity in a further two samples (RI-5 and RI-8, $p<0.05$, less activity than unfiltered sample, but gastrin levels still reduced compared to control), but did not affect tissue gastrin inhibitory activity in the other two samples (RI-3 and RI-4, $p>0.05$, not different to unfiltered samples).

Similarly, centrifugation at 19 500*g* had no effect on tissue gastrin inhibitory activity of rumen fluid or rumen incubates. Centrifugation at 49 500*g* only reduced the amount of tissue gastrin inhibitory activity in one rumen fluid sample (RUM-26) out of seven tested. Centrifugation of rumen incubates at 49 500*g* produced mixed results (shown Tables 5.10A and B). In some cases, centrifugation removed some of the tissue gastrin inhibitory activity (RI-24, b, c, d; RI-25b, c, d; RI-26b, c; RI-27b; RI-29), while in others it did not affect activity (RI-25d, RI-26d, RI-27c and d, RI-28).

Results of testing "breakdown" activity in rumen samples, both untreated and centrifuged at 19 500*g*, kept for 40 days and assayed on four occasions, giving four replicates, are detailed in Table 5.10A. Calculations were conducted in similar fashion to those in Table 5.3, though A and B untreated are not shown. It is noticeable that for all samples, the 'a' and 'b' samples had the same degree of breakdown for all samples ($p>0.05$), but were less than both 'c'

Treatment	UNTREATED		20K CENT	
Sample	*Tissue response*	"breakdown"	*Tissue response*	"breakdown"
RI-24b	-90±1%	-81±1%*	-92±1%	-70±2%**
RI-24c	-87±2%	-94±2%*	-73±1%	-94±1%**
RI-25b	-89±1%	-80±2%*	-92±1%	-79±1%**
RI-25c	-92±2%	-90±3%	-88±1%	-93±2%
RI-26b	-90±1%	-70±1%**	-95±1%	-63±2%**
RI-26c	-92±2%	-91±4%	-88±1%	-89±3%
RI-27b	-88±1%	-73±5%*	-92±1%	-52±7%**
RI-27c	-91±1%	-93±1%	-91±1%	-91±2%

Table 5.11A Comparison between gastrin "breakdown" and tissue gastrin inhibitory activity in four rumen incubates tested twice, untreated and after centrifugation at 19 500*g* (20K cent). All results are expressed as mean±SEM. For each sample, the tissue gastrin inhibitory activity (tissue response) and breakdown are shown, *= $p<0.01$, **= $p<0.001$ B_2 values, an estimate of the breakdown effect, are different from the observed values after incubation with tissue *in vitro.*

Treatment	UNTREATED		50K CENT	
Sample	Tissue response	"breakdown"	Tissue response	"breakdown"
RI-24b	-90±1%	-81±1%*	-53±2%[a]	-54±3%
RI-24c	-87±2%	-94±2%*	-56±3%[a]	-94±2%**
RI-24d	-82±3%	-30±6%	-47±7%[a]	-22±10%*
RI-25b	-89±1%	-80±2%*	-53±2%[a]	-75±2%**
RI-25c	-92±2%	-90±3%	-24±4%[a]	-90±2%**
RI-25d	-85±2%	-37±5%*	-44±6%[a]	-23±7%
RI-26b	-90±1%	-70±1%**	-20±5%[a]	-76±1%*
RI-26c	-92±2%	-91±4%	-15±4%[a]	-89±2%**
RI-26d	-84±2%	-43±10%*	-86±2%	-36±2%**
RI-27b	-88±1%	-73±5%*	-34±3%[a]	-69±5%**
RI-27c	-91±1%	-93±1%	-82±2%	-93±2%*
RI-27d	-89±3%	-41±3%*	-95±1%	-19±6%**
RI-28	-58±11%	-36±8%	-67±4%	-44±4%
RI-29	-72±6%	1±7%	-31±10%[a]	1±4%**

Table 5.11B Comparison between gastrin "breakdown" and tissue gastrin inhibitory activity effect in six rumen incubates, four of which were tested thrice, untreated and after centrifugation at 49 500*g* (50K cent). All results are expressed as mean±SEM. For each sample, the tissue gastrin inhibitory activity (tissue response) and breakdown are shown, *= $p<0.01$, **= $p<0.001$ B_2 values, an estimate of the breakdown effect, are different from the observed values after incubation with tissue *in vitro*, [a]=$p<0.05$ centrifugation reduced inhibitory effect in tissue test.

and 'd' ($p<0.05$), which were also similar ($p>0.05$). Gastrin "breakdown" could account for all the tissue gastrin inhibitory activity in RUM-14a, c, d; RUM-15a to d; RUM-16c, d; and RUM-17a, c, d. In fact for, RUM-14d, RUM-15d, RUM-16c, d and RUM-17d the estimate of "breakdown" was greater than the tissue gastrin inhibitory activity. Centrifugation at 19 500*g* did not result in a significant reduction in "breakdown" activity in any of the samples, though results were close to significance level for RUM-14a. RUM-15a and RUM-15b (p between 0.05 and 0.07), which was enough to cause a significant difference between B_2 and B actual for these samples; before centrifugation they were the same. The reverse was observed for RUM-16a. Centrifugation of samples at 49 500*g* did not affect "breakdown" activity ($p>0.05$), with no difference between B_2 and B actual for these samples (Table 5.10B).

In general, gastrin "breakdown" activity in rumen incubates was not reduced by centrifugation at 19 500*g* ($p>0.05$). Centrifugation at 19 500*g* did reduce "breakdown" effect in RI-24b and RI27b ($p<0.05$), which increased the difference between "breakdown" effect and tissue gastrin inhibitory activity (Table 5.11A).

In general, centrifugation of incubates at 49 500*g* did not affect gastrin "breakdown" activity (Table 5.11B). The exception was RI-25b, where "breakdown" activity was reduced by centrifugation. In contrast, tissue gastrin inhibitory activity was reduced by centrifugation (RI-24, b, c, d; RI-25b, c, d; RI-26b, c; RI-27b; RI-29), such that in many cases gastrin "breakdown" estimate resulted in a significantly lower than the concentration observed in the tissue test (RI-24c; RI-25b, c; RI-26b, c; RI-27b, c). The reverse occurred for RI-26d and RI-27d samples, where tissue gastrin inhibitory activity was high, not reduced by centrifugation, and greater than could be accounted for by "breakdown" activity.

5.3.3.3.5 Effect of protease inhibitor

Addition of a bacterial protease inhibitor at 10mg.mL^{-1} caused an increase in gastrin levels (+71±10%, $p<0.01$) in the tissue test system. The presence of protease inhibitor abolished the tissue gastrin inhibitory activity of two of the three rumen samples tested (converting RUM-24 activity from -57±3% to -13±15% and RUM-25 from -45±5% to 2±7%) but did not affect the

activity of the third rumen sample or the rumen incubate sample. "Breakdown" activity for both rumen fluid and the incubate sample were low, RUM-24 -19±3%, RUM-25 -19±7%, RUM-26 -23±3%, RI-29 -2±8%, despite being greater in rumen fluids in previous tests (-63±6%, -53±6%, -75±4% respectively) being stored at -18°C in between tests. The presence of protease inhibitor did not further reduce "breakdown" activity at any concentration.

5.4 Discussion

Experiments presented in this chapter show that an inhibitor of *in vitro* gastrin secretion exists in rumen fluid, and can also be generated by incubation of abomasal and rumen contents in basal incubation medium. The rumen may therefore be the source of inhibitory activity observed in hypoacidic abomasal contents during parasitism. Incubation of either rumen fluid or abomasal fluid was able to produce inhibitory activity in basal medium. These results show that gastrin inhibitory activity could be produced by bacteria, but is not specific to a particular species.

Rumen fluids and rumen incubates appear to contain three competing factors which affect the *in vitro* tissue assay. These are an inhibitor of gastrin secretion, a stimulant of gastrin secretion and a gastrin elimination, or "breakdown" factor. "Breakdown" of gastrin could account for a significant proportion of the *in vitro* inhibitory activity in rumen fluid, but for less in rumen and abomasal incubates. When "breakdown" activity was removed from samples, this did not necessarily result in an increase in gastrin concentrations in the tissue tests. Furthermore, there were times, even with rumen fluid samples, where "breakdown" activity could not account for the reduction in gastrin concentrations produced in the *in vitro* tissue tests. Therefore, an inhibitor of gastrin secretion does exist in rumen fluid and rumen incubates, but at times is masked by "breakdown" activity.

The gastrin removal activity by rumen fluid or incubate solutions is probably due to either cleavage of the gastrin peptide, or binding of the gastrin peptide such that it cannot be recognised by the RIA gastrin antiserum.

In both rumen fluid and rumen incubate solutions, tissue inhibitory activity, which includes both the gastrin secretion inhibitor and gastrin "breakdown" activity, was removed by low pH and high temperature. There were differences in sensitivities, with incubate inhibitory activity being less sensitive to temperature. The "breakdown" activity in rumen incubates appears to be more sensitive to pH than the inhibitor of gastrin secretion. Generally, filtration and centrifugation did not remove the secretion inhibitor or "breakdown" from rumen contents, but did sometimes reduce or remove the secretion inhibitor from incubates.

5.4.1 Inhibitory activity in rumen contents

All rumen fluids tested contained inhibitory activity (Section 5.3.1). This activity in rumen fluid could be due to two effectors: an inhibitor of gastrin secretion and a "breakdown" effect.

Rumen contents caused between 30% and 90% "breakdown" of gastrin, an effect which was not removed by centrifugation. Calculations were conducted to determine whether the tissue gastrin inhibitory activity could be wholly explained by a "breakdown" effect. These calculations formed an estimate of what the concentration of gastrin would be after incubation with test solutions if "breakdown" were the only effect present in those solutions. As these calculations involved more error due to assumptions in the calculations, the stringency of statistical tests was increased, with responses only considered different where $p<0.01$. Statistical tests showed that there was a significant difference between the estimated "breakdown" effect and the tissue gastrin inhibitory activity, showing that an inhibitory factor that was not "breakdown" existed in rumen fluids.

The estimate of "breakdown" could be considered conservative as it was conducted using non-sulphated human G17. Ovine antral G17 is observed to be up to 80% sulphated (Andersen, 1985). In circulation, sulphation increases the half-life of G17 by up to five times (Pauwels *et al.,* 1987). In addition, at least a small percentage of gastrin released from the G cell is in the G34 form, which is also more resistant to metabolism *in vivo* (Walsh *et al.,* 1974, 1976; Rehfeld and Uvnas-Wallenstein, 1978; Xu and Cranwell, 1992). G34 is also slightly more resistant to degradation than G17 in the pig stomach contents (Xu

et al., 1996). Although the "breakdown" effect may inactivate gastrin by different mechanisms than *in vivo* degradation and unaffected by sulphation of G17 or its extension to G34, it seems unlikely that G34 and sulphated G17 would be more susceptible to degradation than non-sulphated G17.

It is possible that the removal of gastrin by rumen fluid is due to an enzymatic action. Bacterial protease inhibitor used in the present experiments appeared to damage the antral tissue, and thus results were inconclusive. Studies of enzymatic cleavage of gastrin in the stomach and intestine has been studied, but not with rumen contents. Results from pigs (Xu *et al.,* 1996) suggest that gastrin can be broken down in the pig stomach and intestine, possibly due to enzymes produced by the host. Gastrin removal in acidified sheep abomasal contents is rapid (Shulkes *et al.,* 1984), probably due to the actions of pepsin. These results suggest that gastrin is sensitive to degradation by the serine proteases in the small intestine, and pepsin, thus degradation of gastrin by enzymes is possible. There was no degradation of gastrin with abomasal samples in the present experiments. This is probably because pepsin is completely inactive at neutral pH levels at which the present experiments were conducted. Another possibility is that something in the rumen and rumen incubate solutions binds gastrin, making it unavailable to interact with the antiserum in the gastrin RIA. *H. pylori* has recently been demonstrated to have a membrane receptor that binds gastrin at very low concentrations (Chowers *et al.,* 1999). It is possible that a similar peptide exists in rumen bacteria.

When "breakdown" activity appears to account for all of the tissue gastrin inhibitory activity, this does not mean the secretion inhibitor is not present, but rather that it is not possible to quantify this effect. The inhibitory activity may actually be present, but "breakdown" activity masks its presence. This was shown in the repeated tests of RI-24 to RI-27, where "breakdown" activity does not account for tissue inhibitory activity in the a sample tests, but could account for all of the activity in the 'b' and 'c' tests. Thus, an inhibitor of secretion was present in the 'a' and 'd' tests, and was probably also present in the 'b' and 'c' tests, but its effects were masked by increased "breakdown" activity. However, the only cases where the presence of a gastrin secretion inhibitor can be

postulated with any confidence is when the negative response in the tissue test far exceeds "breakdown" activity.

An added complication determining when inhibition of gastrin secretion occurred with rumen fluids was the presence of a stimulant of gastrin secretion. This stimulant of gastrin secretion is sometimes prominent in abomasal fluid (Chapter 4). It was also evident in one filtration (Figure 5.7, RUM-10) and may also be the cause tissue inhibitory activity in response to rumen fluids being less than predicted by the "breakdown" effect (Figure 5.10A and B).

In general, the detection and characterisation of a secretion inhibitor in rumen contents was difficult in the presence of large amounts of "breakdown" activity and a stimulant of gastrin secretion. The presence of a gastrin secretion inhibitor was more obvious in rumen and abomasal incubates as "breakdown" activity generally minimal, between 0 and 20%.

5.4.2 Microbial production of inhibitory activity

The best evidence for a microbial origin of the gastrin secretion inhibitor is the ability to generate such activity with abomasal or rumen incubates. Incubation of abomasal contents in the *in vitro* assay basal medium consistently produced tissue gastrin inhibitory activity, in agreement with results presented by Lawton (1995). In addition, incubation of rumen fluid also produced tissue gastrin inhibitory activity. Production of tissue gastrin inhibitory activity was not specific to the Actinomycete population that predominated in rumen/abomasal incubations, as *S. bovis* also produced this activity. *S. bovis* was able to produce tissue gastrin inhibitory activity more quickly than the Actinomycete culture, probably because of faster proliferation rate. Appearance of activity appeared to be dependent on high bacterial numbers, close to the maximum which could be supported by the medium, and a reduction in medium pH.

Generally, when bacteria were removed from rumen fluids or incubates, at least some tissue gastrin inhibitory activity remained in the bacteria-free solution. This was the case with both centrifugation at 49 500*g* and filtration through a 0.22μm membrane (Section 5.3.3.3.4 and Figure 5.7). Tissue gastrin inhibitory activity in rumen fluid was not due to bacterial modification of the media, as inclusion of an antibiotic mix did not affect the inhibitory action. Thus, it would seem that tissue gastrin inhibitory activity present in both rumen

fluid and rumen incubates is due to the presence of one or several chemical constituents of the solution.

Centrifugation at 49 500*g* was adopted as a method of bacterial removal, as it presented a number of advantages over filtration. It was thought that removal of tissue gastrin inhibitory activity from solutions during filtration may have been a result of the active compounds being hydrophobic and adhering to the filtration membrane. In an attempt to overcome this centrifugation was adopted to remove bacteria. Results showed that centrifugation was just as likely to remove tissue gastrin inhibitory activity from solution as filtration. Centrifugation did, however, have other advantages over filtration in that it was cheaper, faster, required less preparation and was effective with smaller volumes.

Centrifugation at 49 500*g* sometimes removed a significant proportion of tissue gastrin inhibitory activity from rumen and abomasal incubate samples, (Section 5.3.2.2, Table 5.11B), but not from rumen fluids (Figure 5.10B). This suggests that, for incubates, the tissue gastrin inhibitory activity is often associated with large particles and bacteria. Repeated freezing and thawing of solutions sometime dissociated tissue gastrin inhibitory activity from bacteria or the large particles which were removed from solution by centrifugation (compare sample RI-26c to d and RI-27b to c, Table 5.11B). These results suggest that the active inhibitory compounds are not removed by centrifugation due to their own size, but because they are bound to large particles/bacteria. Freezing of samples without a rapid freezing process is likely to result in lysis of bacteria. Thus, after freezing solutions several times, it is likely that enough bacterial damage has occurred for enough inhibitory activity to be liberated and remain in solution after centrifugation.

Repeated freezing and thawing increased “breakdown” activity with both rumen and rumen incubate samples. This was most clearly shown with rumen incubates. The range for “breakdown” activity in rumen and abomasal incubates before repeated freezing and thawing was from 0-60%, though most showed between 0 and 20%. Repeated freezing and thawing increased “breakdown” activity in RI-24 to RI-27 from initially low levels to levels equivalent to the tissue gastrin inhibitory activity (Table 5.4B). However, “breakdown” activity appeared to deteriorate during storage at -18°C, and was

greatly reduced in samples stored for three months (compare RI-24 to RI-27c samples to RI-24 to RI-27d samples).

Centrifugation at 19 500*g* and 49 500*g* was unable to consistently remove the "breakdown" activity from either rumen fluid or rumen incubate samples. Exceptions were centrifugation at 19 500*g* with RI-24b and RI-27b in and at 49 500*g* with RI-27d, which reduced "breakdown" activity, but did not reduce inhibitory activity in tissue tests. These results show the dissociation between the breakdown effect and the *in vitro* tissue inhibition of gastrin secretion, suggesting, these effects are separate.

The inability of centrifugation at 49 500*g* to remove "breakdown" activity, but its ability to sometimes reduce tissue gastrin inhibitory activity, created some anomalous results with rumen incubate samples. In some cases, centrifugation at 49 500*g* resulted in B_2, the gastrin concentration expected in the tissue test after "breakdown" was accounted for, being much less than the observed B value. This was also observed in rumen fluid samples (RUM14 to RUM-17, all d samples, and RUM-16c). Clearly, some tissue inhibitory activity has been removed that is not a "breakdown" effect. It is suggested that the presence of a stimulant, in unison with a reduction of the secretion inhibitor, accounted for greater gastrin concentration in the tissue test than would be predicted by "breakdown." The discrepancy between the predicted and observed results could also suggest that elimination activity is not related to results observed in the tissue experiment.

The presence of a stimulant of gastrin secretion is also observed in incubate samples prior to the appearance of a tissue gastrin inhibitory effect. This stimulatory activity may mask the presence of inhibitory effects, initially at least. Thus, media may contain inhibitory activity earlier than reported. As with rumen fluids, the possible competitive action between the inhibitory and stimulatory gastrin secretion activities complicates attempts to assess the presence and precise sensitivities of the gastrin secretion inhibitor in incubate solutions.

There have been numerous studies of the effects of fermentation products from alcohol production stimulating acid and gastrin release (Singer, 1987, 1991; Teyssen *et al.,* 1993, 1997, 1999). These studies have yet to show which chemical constituent of fermentation is responsible for the

stimulation of gastrin release. None of the studies have reported the presence of a gastrin secretion inhibitor.

Lawton (1995) reported that the inhibition of gastrin secretion by abomasal incubates was linked to an increase in somatostatin secretion. In experiments presented here, there was significant interference with the somatostatin RIA, when incubates contained tissue gastrin inhibitory activity. As such, the effects of incubates on somatostatin could not be determined. This may have been the case in the experiments of Lawton (1995), in which the effects of incubates on the gastrin and somatostatin assays does not appear to have been tested.

5.4.2.1 Sensitivity.

In theory, the pH sensitivity of tissue gastrin inhibitory activity is important for two reasons: firstly to determine when rumen activity is likely to survive in the parasitised abomasum and secondly to determine parameters for separation processes. Whatever the inhibitory chemicals are in rumen contents and rumen incubates, they are sensitive to low pH (Figure 5.4, Table 5.2 and Figure 5.5 Table 5.3 respectively). Removal of tissue gastrin inhibitory activity is maximal at pH2 and minimal at pH5, but responses to pH values between these points varies between samples.

Results with rumen incubates suggest that the secretion inhibitor is less sensitive to pH than the "breakdown" effect. In the four samples where this was tested, tissue gastrin inhibitory activity was always decreased or removed at pH2 and pH3, but was near maximal in two and present in the third at pH3.5 of the four samples (Table 5.7). In contrast, in the one sample where "breakdown" activity was significant, it was much reduced at pH3, 3.5 and 4. However, these results need to be interpreted with caution, as comparisons were only conducted in a few samples, and only one where "breakdown" activity was significant.

Temperature results suggest that the activity in the rumen fluid and rumen incubate are different and that the activity in rumen fluid is temperature sensitive. Temperature sensitivity is different between rumen incubate solutions (Figure 5.6, Table 5.4 and Table 5.5 respectively). Activity in rumen solutions is sensitive to 45°C upwards, while the incubated solutions appear to

tolerate temperatures up to 55°C. These results may be partly due to inaccuracy in the temperature setting. Sensitivity to temperature is likewise important as a consideration for separation procedures but is unlikely to play a role in the survival of activity in the abomasum. Both temperature and acid sensitivity results suggest a protein or polypeptide action is responsible for inhibitory activity.

5.4.3 Survival of rumen activity in the hypoacidic abomasum

The rumen does contain an inhibitor of *in vitro* gastrin secretion, and thus could be the source of the inhibitory activity appearing in the hypoacidic abomasum. The activity in rumen contents, and in rumen incubates is sensitive to acidity in the range of pH2 and pH3. Thus, in the unparasitised animal, the inhibitory activity present in the rumen would usually be destroyed by the low pH environment of the abomasum, but may survive in the hypoacidic abomasum during parasitism. It is noticeable that rumen fluids tested in experiments presented here contain significant amounts of gastrin "breakdown" activity, unlike the abomasal samples tested in Chapter 4. This may be due to "breakdown" activity being more sensitive to acidity than the gastrin secretion inhibitor, as was shown with RI-27d (Table 5.7). This explanation may not explain why no inhibitory activity was present in abomasal samples over pH6 (Table 4.3).

In the next chapter, experiments are described in which separation of the activity in the rumen fluid and rumen incubates was attempted. The techniques employed were based on consideration of the properties of activity revealed in this chapter and Chapter 4. In Chapter 4, it was shown that tissue gastrin inhibitory activity in rumen contents is present at low concentrations. This was shown by the necessity for rumen contents to make up 5-20% of the tissue incubation medium. Thus, high volume, high yield techniques are necessary, for the initial separation process at least. In experiments presented here it has been shown that inhibitory activity is sensitive to acidic conditions, below pH3, and possibly also temperature. This must also be considered in separations.

5.5 Summary

Results from all experiments suggest that there may be three competing factors in rumen fluid and rumen incubates which affect the *in vitro* tissue assay results. These are an inhibitor of gastrin secretion, a gastrin elimination factor and a stimulant of gastrin secretion. Detection of the secretion inhibitor can be masked by both the secretion stimulant and the gastrin "breakdown" activity. This clouds interpretation of inhibitory results in tissue tests.

There is a significant amount of "breakdown" activity in rumen fluids, which can account for 30-90% of the tissue gastrin inhibitory activity. The amount of "breakdown" activity in rumen and abomasal incubates is considerably less, accounting for 0-60% of the tissue gastrin inhibitory activity. The inhibitor of gastrin secretion and the gastrin breakdown effect alike appear to be sensitive to pH levels of 2-3. The sensitivity of activity to temperature was unclear, being sensitive to 45°C and above in rumen fluid and insensitive to temperatures up to 55°C in incubates. Both the inhibitor of gastrin secretion and the "breakdown" effect are also associated with the bacteria, but can be separated from bacteria using either filtration and centrifugation. Repeated freezing and thawing of samples liberates the gastrin "breakdown" effect from bacteria, so that it becomes more prevalent in the test system. There is also a stimulant of gastrin secretion present in solutions, which is evident in incubates prior to the appearance of inhibitory activity, and on occasions where elimination is removed from solution.

Despite the presence of other masking factors in solutions, there is enough evidence to suggest that a gastrin secretion inhibitor does exist, in rumen solutions. Thus, the rumen could be the source of the inhibitor of *in vitro* gastrin secretion observed in the hypoacidic abomasal contents of parasitised sheep.

CHAPTER 6

SEPARATION OF *IN VITRO* INHIBITORY ACTIVITY FROM RUMEN CONTENTS AND INCUBATED SOLUTIONS

6.1 Introduction

Rumen fluids, hypoacidic abomasal fluids and incubates of rumen and abomasal fluids all contain inhibitors of *in vitro* gastrin secretion (shown in Chapters 4 and 5). This inhibitor of *in vitro* gastrin secretion is sensitive to acidic pH, between pH2 and 3 (Chapter 5) and possibly also temperatures above 55°C, suggesting that the active compounds are peptides or proteins. This is hypothesised because other types of compound tend to be less sensitive to irreversible degradation by low pH and temperature.

Experiments in previous chapters suggest that concentrations of the active substances are quite low: rumen fluid had to make up 5% of the final solution volume before it produced an effect on tissue. Thus, high volume, high yield techniques are probably the most appropriate, for the initial separation process at least. Solubility, polarity and size were not clear from previous work. Determining these were the aims of the separation.

The most limiting complication with separation of active compounds was the use of biological tissue in the assay system. Maintenance of low salt concentration, neutral pH and reduction of toxicity were central features in deciding what separation techniques were to be used.

In this chapter, experiments aimed at isolating and characterising the compound, or compounds, that have secretion inhibition effects are described. Ultramembrane filtration was used in an attempt to separate activity on the basis of size, as this was shown to be complicated by aggregation and loss of inhibitory activity. Solvents were added to solutions in an effort to aid separation, and their effects on *in vitro* gastrin secretion are described. Finally, procedures using hydrophobic interaction chromatography are described, as

this technique has been effective in the past in separating hydrophobic peptides (Alpert, 1988).

6.2 Methods

6.2.1 Rumen fluid and rumen and abomasal incubates

Rumen fluids were collected on seven occasions from pasture-fed animals for ultramembrane filtrations. Samples were obtained by Massey University staff from a flock of 10 animals with rumen fistulae, though which animals samples were taken from on a particular day was not recorded. Of the samples used for testing:

RUM-27, RUM-28, RUM-29, RUM-33 (rumen samples 27, 28, 29, 33): Each sample was from an individual animal.

RUM-30, RUM-31, RUM-32 (rumen samples 30, 31, 32): Each sample was a mixture of rumen fluids from four animals, all collected on the same day.

Rumen incubates used for separations were produced in the following manner.

RI-3 *to* RI-8 (*rumen incubates* 3 *to* 8): Rumen samples from six pasture-fed animals were diluted 500-fold and incubated at 37°C for 40 hours in normal basal medium.

RI-9/RI-12 (rumen incubate solution containing RI-9 and RI-12): Produced as for RI-3 to RI-8 except solutions were incubated for 70 hours. These samples were stored at -18°C for one month prior to use in separation experiments.

RI-22, RI-23 and RI-30 (rumen incubates 22 and 23): Produced as described for RI-3 to RI-8 except solutions were incubated for 48 hours.

AI-10 (abomasal incubate 10): An abomasal sample from a sheep parasitised with larval *O. circumcincta* was diluted 500-fold in basal medium and incubated at 37°C for 48 hours. Abomasal sample prior to incubation had a pH of 3.65.

6.2.2 Preparation of solutions

RI-3 to RI-8 and RI-9/RI-12 were filtered through 0.22μm filters, while all rumen fluids, RI-22, RI-23, RI-30 and AI-10 were centrifuged at 49 500*g* to

remove all bacteria from solution. Filtration though 0.22μm membranes was initially conducted as described in Section 5.2.3.4; a prefilter (Millipore, AW0314250) and then a 0.22μm hydrophilic filter (Millipore, GVWP142 50) were placed in a pressurised system and solutions pushed through the filters using N_2. Centrifugation was at 49 500*g* as described in Section 5.2.3.5.

6.2.3 Ultramembrane filtration

Ultramembrane filtrations of rumen fluids and rumen incubates were conducted using an Amicon (Amicon Pharmaceuticals, USA) pressure filtration system, which used N_2 gas to push solutions through the filter. The solution was placed in a pressurised stirred cell unit, which kept the solution mixed during filtration in an attempt to stop fluid layers and precipitates forming. Ultrafiltration membranes used were Amicon YM3, with a molecular weight cut-off of 3000M_r (RUM-27 to RUM-32,RI-3 to RI-8 and RI-30), YM10 with a molecular weight cutoff of 10 000M_r (RUM-29 to RUM-33, RI-3 to RI-6, RI-8 and RI-30), YM30 with a molecular weight cutoff of 30 000M_r (RUM-29 to RUM-33, RI-3 to RI-8 and RI-30) and XM50 with a molecular weight cut-off of 50 000M_r (RUM-29, RUM-30, RUM-32 and RUM-33). Each ultramembrane filter was used for several separations and was stored in 5% alcohol when not in use.

Aliquots of RUM-32 were also adjusted to pH5 or pH10 prior to ultramembrane filtration in one experiment. On one occasion, RI-3 was adjusted to pH5 or pH10 and filtered through YM3, YM10 and YM30 filters.

6.2.3.1 Use of salts and solvents in separation

In attempts to stop precipitate formation and/or loss of activity due to membrane binding detergents, salts or solvents were added to the solution. These salts or solvents were tested for their effect on gastrin secretion. If the solvent or salt did not affect basal gastrin secretion, it was added to rumen fluid or rumen incubate prior to ultramembrane filtration, or used to wash filter membranes after filtration.

Effects of non-ionic detergent on the *in vitro* test tissue were examined. Detergents used were: Triton X-100 (BDH Chemicals, UK) at 0.1%, 0.05%, 0.01% and 0.001% (n=2) of final solution; Nonidet (BDH Chemicals, UK) at 1%,

0.1%, 0.01% and 0.001% (n=2); Tween 80 (BDH Chemicals, UK) at 1%, 0.1%, 0.05%, 0.01%, and 0.001% (n=2); Thesit (P9641, Sigma, USA) at 0.0001% (n=2).

On one occasion, YM3, YM10 and YM30 membranes were soaked in a 0.001% Thesit detergent solution prior to filtration of RI-9/RI-12 in an attempt to stop activity adhering to the membranes.

The effects of hydrophobic solvents on the *in vitro* tissue test were also examined. Solvents tested were added to basal medium in the concentrations specified. Solvents included alcohol (n=2) at 10% (v/v), glycerol (G6279, Sigma, USA) (n=2) at 10% (v/v) and acetic acid (BDH Chemicals, UK) (n=4) at up to 2% (v/v). For *in vitro* testing, 2% acetic acid test solutions were brought to pH7.40±0.02 using 1M NaOH.

Glycerol was added to make up 10% (v/v) of a solution comprising approximately equal amounts of RI-9 and RI-12 prior to ultramembrane filtration through YM3, YM10 and YM30 membranes.

Acetic acid was added to make up 10% (v/v) to rumen filtrate solutions prior to ultramembrane filtration through YM3, YM10 and YM30 membranes. Prior to adding to test solutions, acetic acid (BDH Chemicals, UK) was neutralised to pH4.5 using 6M NaOH, making a 70% v/v acetic acid solution. The 70% v/v acetic acid was added to test solution at a ratio of 1 part per 2.5 parts, resulting in a test solution with 20% v/v acetic acid. Test solutions formed 10% of the final volume of test incubation solution, which therefore contained 2% acetic acid. Acetic acid was added to RI-3 prior to filtration on two separate occasions, and on one occasion to an incubate solution containing approximately equal amounts of RI-3, RI-5 and RI-7. In the two experiments where RI-3 filtrations were tested, solutions were not diluted to account for higher osmolarity. In the test the incubate solution composed of RI-3, RI-5 and RI-7, osmolarity of solutions was lowered to 320-330mOsmol by diluting basal medium.

Salts used were ammonium sulphate, and later sodium sulphate for hydrophobic interaction chromatography and NaCl.

On two separate occasions, RUM-32 had 1M NaCl added and was then filtered through YM10 and YM30 filters.

6.2.3.1.1 Ultramembrane filter wash

In some experiments, it appeared that active compounds in test solutions were adhering to the filter papers. An attempt was made to remove activity from a filter disk by soaking in solvents. One YM10 filtering disk, which previously had been used to filter seven rumen incubate samples, was cut into four parts and each part soaked in either 20% acetic acid, 20% alcohol, NaOH solution at pH12, with the last soaked in basal medium and scraped with a spatula.

6.2.4 Dialysis

A 10mL incubate solution, containing approximately equal amounts RI-3, RI-5 and RI-7, was dialysed in 40mL of basal medium (described in Section 2.2.1) using dialysis tubing with molecular weight cutoff of 14000M_r.

6.2.5 Hydrophobic interaction chromatography

Hydrophobic interaction chromatography was conducted using a fast flow Phenyl superose column at a flow rate of 2mL.min^{-1} connected to an automated system. The column was loaded with test solutions containing 1M Na_2SO_4, 0.01M Na_2HPO_4. The principle of the elution was to gradually reduce the concentration of Na_2SO_4 in the column from 1M to zero. This was done by starting the elution with a solution containing 1M Na_2SO_4, 0.01M Na_2HPO_4, and gradually mixing the solution with 0.01M Na_2HPO_4 solution and adding to the column. At the end of the elution, the column was washed with 0.5M acetonitrile to remove any compounds that were still bound to the column.

Four runs of different solutions were separated on the column. In the first run, 4mL of RI-22, which contained only *in vitro* inhibitory activity (Table 5.7), was applied to the column. RI-22 was freeze dried reducing the water content of the solution 66% in an attempt to concentrate activity.

In the second run, a 20mL sample of RI-23 was added. In the third run, 20mL RUM-26 was added to the column. In the fourth run, 50mL of AI-9 was added to the column. Prior to separation, RI-23, RUM-26 and AI-9 contained little “breakdown” activity (4±4%, -19±7% and -6±3% respectively).

After the first, second and third run was completed, a blank run of buffers was passed through the column without sample. Fractions from the blank run were then tested along with the separation fractions as a control.

6.2.6 Statistical analyses

Statistical analysis was tested using UNIANOVA in SPSS version 9.0. All data were examined for normality using Shapiro-Wilks tests. Data sets in comparisons were tested for equality of variation using Levene's Homogeneity of Variation test. *Post hoc* comparisons of data groups when variances were equal was conducted using Tukey's HSD test for comparisons of all groups, or Dunnett's test when compared with control alone. When variances were unequal, Tamhane's T2 *post hoc* test was used. In cases where data were not normally distributed, Kruskal Wallis analysis in Graphpad Prism version 2.01, with Dunn's *post hoc* test was used. In comparisons of only two data sets, t-tests were conducted in SPSS version 9.0, with data sets again tested by for normal distribution and equality of variance.

6.3 Results

6.3.1 Ultramembrane filtrations

Retention of material by the YM3, YM10, YM30 and XM50 membranes was shown by the different colours of filtrates and retentates. Original rumen solutions were all a clear khaki green tending to brown colour. Filtration of rumen solutions resulted in formation of a dark brown retentate solution which often contained a precipitate, which was also present in the filter wash. The precipitate was grey coloured and formed large flakes. These could be dissolved over time in basal medium and water. Filtrates were a more pale-green colour than original solutions. Filtration of rumen incubates also resulted in an intensification of phenol red colour in the retentate and reduction in colour in the filtrate.

Sample	Treat	Response	sig vs orig	sig vs cont
RUM-27	Orig	-58±1%	-	<0.01
RUM-27	Filt	+113±14%	<0.001	<0.001
RUM-27	Ret	-10±14%	<0.05	ns
RUM-27	FW	+80±14%	<0.001	<0.001
RUM-28	Orig	-76±1%	<0.001	<0.01
RUM-28	Filt	+64±9%	<0.001	ns
RUM-28	Ret	-44±7%	ns	<0.01
RUM-28	FW	+31±18%	<0.001	ns
RUM-29	Orig	-39±1%	-	<0.05
RUM-29	Filt	+2±14%	<0.05	ns
RUM-29	Ret	-61±6%	<0.001	<0.001
RUM-29	FW	-13±12%	ns	ns
RUM-30	Orig	+6±14%	-	ns
RUM-30	Filt	+66±19%	<0.05	<0.05
RUM-30	Ret	-5±1%	ns	ns
RUM-30	FW	+45±11%	ns	ns
RUM-31	Orig	-30±1%	-	<0.05
RUM-31	Filt	+68±1%	<0.001	<0.001
RUM-31	Ret	+21±10%	<0.01	ns
RUM-31	FW	-9±1%	ns	ns
RUM-32	Orig	-39±1%	-	<0.05
RUM-32	Filt	-9±10%	ns	ns
RUM-32	Ret	-79±1%	<0.001	<0.001
RUM-32	FW	-46±1%	ns	<0.01

Table 6.1. Effect of YM3 ultramembrane filtration on the tissue gastrin inhibitory activity of six bacteria free rumen solutions. Shown are the tissue gastrin inhibitory activities (Response) of the rumen solution (mean±SEM) without any filtration (orig), the effect of the filtrate (filt), the effect of the retentate (ret) and the effect of the solution used to wash the filter (FW). All filtration samples are compared to the original effect (sig vs orig) and compared with the control solution for that experiment (sig vs cont).

Sample	Treat	Response	sig vs orig	sig vs cont
RUM-29	Orig	-39±1%	-	<0.01
RUM-29	Filt	+4±17%	ns	ns
RUM-29	Ret	-65±1%	ns	<0.001
RUM-29	FW	-47±1%	ns	<0.001
RUM-30	Orig	+6±15%	-	ns
RUM-30	Filt	+22±14%	ns	ns
RUM-30	Ret	-45±1%	<0.05	<0.05
RUM-30	FW	+56±15%	<0.05	<0.01
RUM-31	Orig	-30±1%	-	<0.05
RUM-31	Filt	+60±10%	<0.001	<0.01
RUM-31	Ret	-73±1%	<0.05	<0.05
RUM-31	FW	-30±1%	<0.05	<0.05
RUM-32	Orig	-39±1%	-	<0.05
RUM-32	Filt	-18±1%	ns	ns
RUM-32	Ret	-58±1%	ns	<0.001
RUM-32	FW	-56±1%	ns	<0.01
RUM-33	Orig	-53±1%	-	<0.001
RUM-33	Filt	-79±1%	<0.001	<0.001
RUM-33	Ret	-91±1%	<0.001	<0.001
RUM-33	FW	-93±1%	<0.001	<0.001

Table 6.2. Effect of YM10 ultramembrane filtration on the tissue gastrin inhibitory activity of five bacteria free rumen solutions. Numbering of samples carries over from table 6.1. Shown are the tissue gastrin inhibitory activities (Response) of the rumen solution (mean±SEM) without any filtration (orig), the effect of the filtrate (filt), the effect of the retentate (ret) and the effect of the solution used to wash the filter (FW). All filtration samples are compared to the original effect (sig vs orig) and compared to the control solution for that experiment (sig vs cont).

Sample	Response	Effect	sig vs orig	sig vs cont
RUM-29	Orig	-39±1%	-	<0.01
RUM-29	Filt	-21±1%	ns	<0.05
RUM-29	Ret	-45±1%	ns	<0.001
RUM-29	FW	-14±1%	ns	ns
RUM-30	Orig	+6±14%	-	ns
RUM-30	Filt	+65±14%	<0.001	<0.001
RUM-30	Ret	-28±1%	ns	ns
RUM-30	FW	+36±1%	ns	<0.05
RUM-31	Orig	-30±1%	-	<0.05
RUM-31	Filt	+46±14%	<0.01	ns
RUM-31	Ret	-69±1%	<0.001	<0.001
RUM-31	FW	-22±4%	ns	ns
RUM-32	Orig	-39±1%	-	<0.05
RUM-32	Filt	+7±15%	ns	ns
RUM-32	Ret	-88±1%	<0.01	<0.001
RUM-32	FW	-71±1%	<0.05	<0.001
RUM-33	Orig	-53±1%	-	<0.001
RUM-33	Filt	-43±1%	<0.001	<0.001
RUM-33	Ret	-67±1%	ns	<0.001
RUM-33	FW	-13±1%	<0.001	ns

Table 6.3. Effect of YM30 ultramembrane filtration on the tissue gastrin inhibitory activity five bacteria free rumen solutions. Numbering of samples carries over from table 6.1. Shown are the tissue gastrin inhibitory activities (Response) of the rumen solution (mean±SEM) without any filtration (orig), the effect of the filtrate (filt), the effect of the retentate (ret) and the effect of the solution used to wash the filter (FW). All filtration samples are compared to the original effect (sig vs orig) and compared to the control solution for that experiment (sig vs cont).

Sample	Treat	Response	sig vs orig	sig vs cont
RUM-29	Orig	-39±1%	-	<0.001
RUM-29	Filt	-16±1%	<0.05	ns
RUM-29	Ret	-41±1%	ns	<0.001
RUM-29	FW	-72±1%	<0.01	<0.001
RUM-30	Orig	+6±14%	-	ns
RUM-30	Filt	+41±14%	ns	<0.05
RUM-30	Ret	-20±1%	ns	ns
RUM-30	FW	+47±10%	ns	<0.01
RUM-32	Orig	-39±1%	-	<0.05
RUM-32	Filt	-19±1%	ns	ns
RUM-32	Ret	-73±1%	<0.05	<0.001
RUM-32	FW	-46±1%	ns	<0.001
RUM-33	Orig	-53±1%	-	<0.001
RUM-33	Filt	-93±1%	<0.001	<0.001
RUM-33	Ret	-93±1%	<0.001	<0.001
RUM-33	FW	-34±1%	ns	<0.05

Table 6.4. Effect of XM50 ultramembrane filtration on the tissue gastrin inhibitory activity of four bacteria free rumen solutions. Numbering of samples carries over from table 6.1. Shown are the tissue gastrin inhibitory activities (response) of the rumen solution (mean±SEM) without any filtration (orig), the effect of the filtrate (filt), the effect of the retentate (ret) and the effect of the solution used to wash the filter (FW). All filtration samples are compared to the original effect (sig vs orig) and compared to the control solution for that experiment (sig vs cont).

Sample	Treat	Response	sig vs orig	sig vs cont
RI-3	Orig	-81±1%	-	<0.001
RI-3	Filt	+4 ±1%	<0.001	<0.05
RI-3	Ret	+11±1%	<0.001	ns
RI-3	FW	+18±1%	<0.001	ns
RI-4	Orig	-85±1%	-	<0.001
RI-4	Filt	+69±1%	<0.001	<0.01
RI-4	Ret	+9±1%	<0.001	ns
RI-4	FW	+24±14%	<0.001	ns
RI-5	Orig	-36±1%	-	<0.05
RI-5	Filt	+57±12%	<0.001	<0.001
RI-5	Ret	+16±1%	<0.01	ns
RI-5	FW	+90±10%	<0.001	<0.001
RI-6	Orig	+22±10%	-	ns
RI-6	Filt	+50±11%	ns	<0.05
RI-6	Ret	-14±1%	ns	ns
RI-6	FW	1±14%	ns	ns
RI-7	Orig	+18±1%	-	ns
RI-7	Filt	+123±1%	<0.001	<0.001
RI-7	Ret	+16±10%	ns	ns
RI-7	FW	+123±11%	<0.001	<0.001
RI-8	Orig	-69±1%	-	<0.001
RI-8	Filt	+44±14%	<0.001	ns
RI-8	Ret	-1±12%	<0.001	ns
RI-8	FW	+20±12%	<0.001	ns
RI-30	Orig	+5±1%	-	ns
RI-30	Filt	+75±1%	<0.001	<0.001
RI-30	Ret	+31±1%	<0.05	<0.01
RI-30	FW	+41±1%	<0.01	<0.001

Table 6.5. Effect of YM3 ultramembrane filtration on the tissue gastrin inhibitory activity in seven bacteria free rumen incubate solutions. Shown are the tissue gastrin inhibitory activities (response) of the incubate solution (mean±SEM) without any filtration (orig), the effect of the filtrate (filt), the effect of the retentate (ret) and the effect of the solution used to wash the filter (FW). All filtration samples are compared to the original effect (sig vs orig) and compared to the control solution for that experiment (sig vs cont).

Sample	Treat	Response	sig vs orig	sig vs cont
RI-3	Orig	-81±1%	-	<0.001
RI-3	Filt	+46±1%	<0.001	<0.001
RI-3	Ret	-14±1%	<0.001	ns
RI-3	FW	+36±1%	<0.001	<0.05
RI-4	Orig	-85±1%	-	<0.001
RI-4	Filt	+33±10%	<0.001	ns
RI-4	Ret	+16±1%	<0.001	ns
RI-4	FW	+47±14%	ns	ns
RI-5	Orig	-36±1%	-	<0.001
RI-5	Filt	+65±1%	<0.001	<0.001
RI-5	Ret	+25±1%	<0.001	ns
RI-5	FW	+111±10%	<0.001	<0.001
RI-6	Orig	+22±10%	-	ns
RI-6	Filt	+10±1%	ns	ns
RI-6	Ret	+94±10%	<0.001	<0.001
RI-6	FW	+37±10%	ns	ns
RI-8	Orig	-69±1%	-	<0.001
RI-8	Filt	+69±12%	<0.001	<0.01
RI-8	Ret	+78±23%	<0.001	ns
RI-8	FW	+23±6%	<0.001	ns
RI-30	Orig	5%1	-	ns
RI-30	Filt	11+1%	ns	ns
RI-30	Ret	+6+13%	ns	ns
RI-30	FW	+19+1%	ns	ns

Table 6.6. Effect of YM10 ultramembrane filtration on the tissue gastrin inhibitory activity of six bacteria free rumen incubate solutions. Numbering of samples carries over from table 6.5. Shown are the tissue gastrin inhibitory activities (Response) of the incubate solution (mean±SEM) without any filtration (orig), the effect of the filtrate (filt), the effect of the retentate (ret) and the effect of the solution used to wash the filter (FW). All filtration samples are compared to the original effect (sig vs orig) and compared to the control solution for that experiment (sig vs cont).

Sample	Treat	Response	sig vs orig	sig vs cont
RI-3	Orig	-81±1%	-	<0.001
RI-3	Filt	+54±12%	<0.001	<0.001
RI-3	Ret	-17±1%	<0.001	ns
RI-3	FW	-60±1%	<0.001	<0.001
RI-4	Orig	-85±1%	-	<0.001
RI-4	Filt	+45±1%	<0.001	ns
RI-4	Ret	+66±1	<0.001	<0.01
RI-4	FW	+34±24%	<0.001	ns
RI-5	Orig	-36±1%	-	<0.001
RI-5	Filt	+29±1%	<0.001	ns
RI-5	Ret	+49±1%	<0.001	<0.05
RI-5	FW	+131±30%	<0.001	<0.05
RI-6	Orig	+22±7%	-	ns
RI-6	Filt	+97±29%	ns	ns
RI-6	Ret	-8±1%	ns	ns
RI-6	FW	+109±1%	<0.001	<0.001
RI-7	Orig	+19±1%	-	ns
RI-7	Filt	+4±1%	ns	0.01
RI-7	Ret	+8±1%	ns	ns
RI-7	FW	+156±32%	ns	<0.05
RI-8	Orig	-69±1	-	<0.001
RI-8	Filt	+23±11%	<0.001	ns
RI-8	Ret	+99±13%	<0.001	<0.001
RI-8	FW	+28±13%	<0.001	ns
RI-30	Orig	+5±1%	-	ns
RI-30	Filt	+29±12%	ns	ns
RI-30	Ret	+11±1%	ns	ns
RI-30	FW	+20±1%	ns	ns

Table 6.7. Effect of YM30 ultramembrane filtration on the tissue gastrin inhibitory activity of seven bacteria free rumen incubate solutions. Numbering of samples carries over from table 6.5. Shown are the tissue gastrin inhibitory activities (response) of the incubate solution (mean±SEM) without any filtration (orig), the effect of the filtrate (filt), the effect of the retentate (ret) and the effect of the solution used to wash the filter (FW). All filtration samples are compared to the original effect (sig vs orig) and compared to the control solution for that experiment (sig vs cont).

Sample	Treat	Response	orig	sig vs orig
RUM-27	YM3	-10±14%	-58±1%	<0.05
RUM-28	YM3	-44±7%	-76±1%	ns
RUM-29	YM3	-61±6%	-39±1%	<0.001
RUM-29	YM10	-65±1%	-39±1%	ns
RUM-29	YM30	-45±1%	-39±1%	ns
RUM-29	XM50	-41±1%	-39±1%	ns
RUM-30	YM3	-5±1%	+6±14%	ns
RUM-30	YM10	-45±1%	+6±14%	<0.05
RUM-30	YM30	-28±1%	+6±14%	ns
RUM-30	XM50	-20±1%	+6±14%	ns
RUM-31	YM3	+21±10%	-30±1%	<0.01
RUM-31	YM10	-73±1%	-30±1%	<0.05
RUM-31	YM30	-69±1%	-30±1%	<0.001
RUM-32	YM3	-79±1%	-39±1%	<0.001
RUM-32	YM10	-58±1%	-39±1%	ns
RUM-32	YM30	-88±1%	-39±1%	<0.01
RUM-32	XM50	-73±1%	-39±1%	<0.05
RUM-33	YM10	-91±1%	-79±1%	<0.001
RUM-33	YM30	-67±1%	-79±1%	ns
RUM-33	XM50	-93±1%	-79±1%	<0.001

Table 6.8. Tissue gastrin inhibitory activity in retentate solutions from six rumen fluid solutions (RUM-27 to RUM-33) after filtration through YM3, YM10, YM30 or XM50 ultramembranes. Shown are the tissue gastrin inhibitory activity (Response) of the rumen solution (mean±SEM) without any filtration (orig), and in retentate (effect) after filtration through the described ultramembrane (response) . All retentate samples are then compared to the original samples (sig vs orig).

Sample	Treat	Response	orig	sig vs orig
RI-3	YM3	+11±1%	-81±1%	<0.001
RI-3	YM10	-14±1%	-81±1%	<0.001
RI-3	YM30	-17±1%	-81±1%	<0.001
RI-4	YM3	+9±1%	-85±1%	<0.001
RI-4	YM10	+16±1%	-85±1%	<0.001
RI-4	YM30	+66±1	-85±1%	<0.001
RI-5	YM3	+16±1%	-36±1%	<0.01
RI-5	YM10	+25±1%	-36±1%	<0.001
RI-5	YM30	+49±1%	-36±1%	<0.001
RI-6	YM3	-14±1%	+22±10%	ns
RI-6	YM10	+94±10%	+22±10%	<0.001
RI-6	YM30	-8±1%	+22±10%	ns
RI-7	YM3	+16±10%	+19±1%	ns
RI-7	YM30	+8±1%	+19±1%	ns
RI-8	YM3	-1±12%	-69±1%	<0.001
RI-8	YM10	+78±23%	-69±1%	<0.001
RI-8	YM30	+99±13%	-69±1%	ns
RI-30	YM3	+31±1%	+5±1%	<0.05
RI-30	YM10	+11±1%	+5±1%	ns
RI-30	YM30	+11±1%	+5±1%	ns

Table 6.9. Tissue gastrin inhibitory activity in retentate solutions from seven rumen incubate solutions (RI-3 to RI-30) after filtration through YM3, YM10, YM30 or XM50 ultramembranes. Shown are the Tissue gastrin inhibitory activities (Response) of the rumen solution (mean±SEM) without any filtration (orig), and in retentate (response) after filtration through the described ultarmembrane (treat) . All retentate samples are then compared to the orginal samples (sig vs orig).

6.3.1.1 Ultramembrane filtration of rumen fluids

The *in vitro* gastrin response to the fractions of ultramembrane filtration of rumen fluids through YM3, YM10, YM30 and XM50 membranes is shown in Tables 6.1-6.4, and to filtration of rumen incubates in Tables 6.5-6.7.

For most samples, both rumen fluids and rumen incubates, no inhibitory activity was present in any of the filtrates. The filtrates, particularly the rumen incubates and the YM3 filtrates of rumen fluids, stimulated gastrin secretion.

Only filtrates of RUM33 consistently contained inhibitory activity. The YM30 filtrate of RUM-29 also contained inhibitory activity, but not the XM50 filtrate. The flow rate of RUM-29 through all membranes was faster than observed for all other samples, possibly due to incorrect sealing of the filter chamber. Although some separation of contents did occur for RUM-33 filtration, as shown by the lighter colour filtrates and darker retentates, exclusion of large molecules from filtrates may not have been complete.

Activity in all the retentates after ultramembrane filtration are summarised for rumen fluids in Table 6.8 and for rumen incubates in Table 6.9. For rumen fluids, there was inhibitory activity in the retentate solutions. This was not the case for rumen incubates, which lost any inhibitory activity in ultramembrane filtration, although retentates tended to stimulate gastrin secretion less than filtrates. Inhibitory activity was also lost after filtration of RUM-27 and RUM-31 through YM3. Concentration of retentate activity was dependent on the degree of ultrafiltration of the sample. For example, RUM-29, RUM-30 and RUM-31 were allowed to filter more solution for YM10 than for YM3, while for sample RUM-32 was more completely filtered through YM3 than through YM10.

Filter washes after ultramembrane filtration of rumen fluids had variable effects on gastrin secretion, and contained variable amounts of precipitate. RUM-32 had significant inhibitory effects, while for RUM-27, the filter wash had significant stimulatory effects. There were marked gastrin stimulatory effects in the filter washes of RI-5, RI-6 and RI-7 also. However, the osmotic pressure of these samples was low, between 260 and 280mOsmL^{-1}.

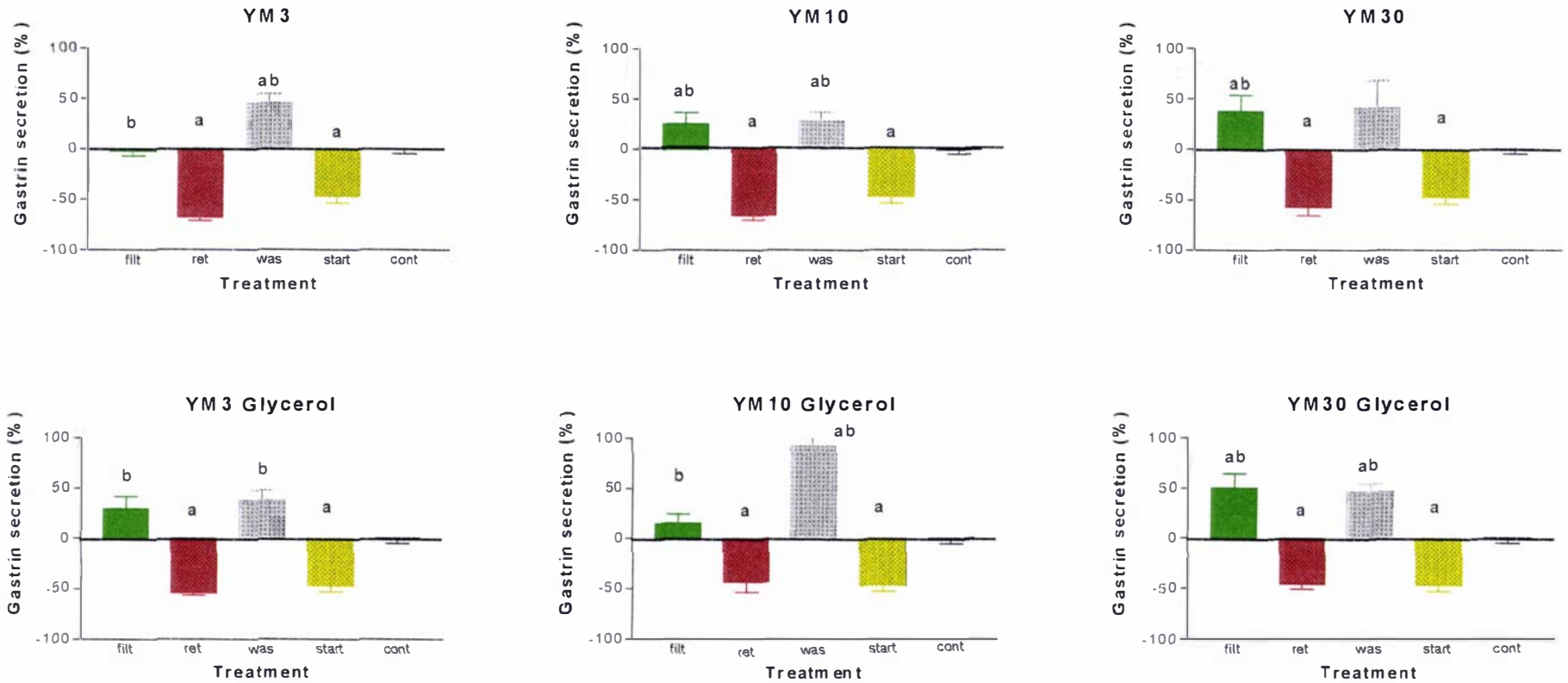

Figure 6.1. Ultramembrane filtration of a rumen incubate sample through YM3, YM10 and YM30 ultramembrane alone and with 10% glycerol (v/v) added before filtering. The top three graphs (YM3, YM10, YM30) show the effects of ultramembrane filtration of rumen contents alone while the bottom three (YM3, 10 and 30 Glycerol) show the effect of adding glycerol to the test solution prior to filtering. Treatment groups are filt= filtered, ret= retentate, was= filter wash, start= starting test solution, cont= control solution. a= treatment group is different to control, b= treatment group is different to the starting active solution.

Soaking filters in 20% alcohol, 20% acetic acid or in NaOH solution at pH12, or scraping the filter basal media did not result in extraction of any inhibitory activity from the filter paper.

Increasing or decreasing pH did not affect filtration properties of either rumen fluids or rumen incubates. Addition of NaCl to RUM-32 did not result in activity passing through any filters, but did prevent aggregation in the retentate.

In none of the three experiments where acetic acid was added to rumen incubates did activity appear in the filtrate solution. Addition of 10% glycerol to an active incubate solution (RI-9/RI-12) did not result in inhibitory activity passing through YM3, YM10 or YM30 filters (Figure 6.1). Unlike RI-3 to RI-8 filtered through YM3, YM10 and YM30 filters RI-9/RI-12 did contain inhibitory activity in retentates. Unlike RI-3 to RI-8, RI-9/RI-12 was stored at -18°C prior to filtration through 0.22μm, removing bacteria. T-tests did reveal some differences between means for samples where glycerol was added versus samples without, but this related to the degree of response and not to whether the filter excluded activity or not.

6.3.2 Dialysis

A mixed incubate solution containing RI-3, RI-5 and RI-7 had significant inhibitory effects on gastrin secretion (-44±1%, $p<0.01$) prior to dialysis. After dialysis, there was a complete loss of activity from both the contents of the dialysis bag (gastrin response -5±3% $p>0.05$ not different to control) and the dialysate (gastrin response -2±6%, $p>0.05$, not different to control).

6.3.3 Effect of salts, solvents and detergents on *in vitro* gastrin secretion

6.3.3.1 Hydrophobic solvents

Ethanol and glycerol at 2% concentration did not affect basal gastrin secretion (+13±5% and +6±6% respectively). The presence of acetic acid in the tissue test system caused inhibition of gastrin secretion when solutions

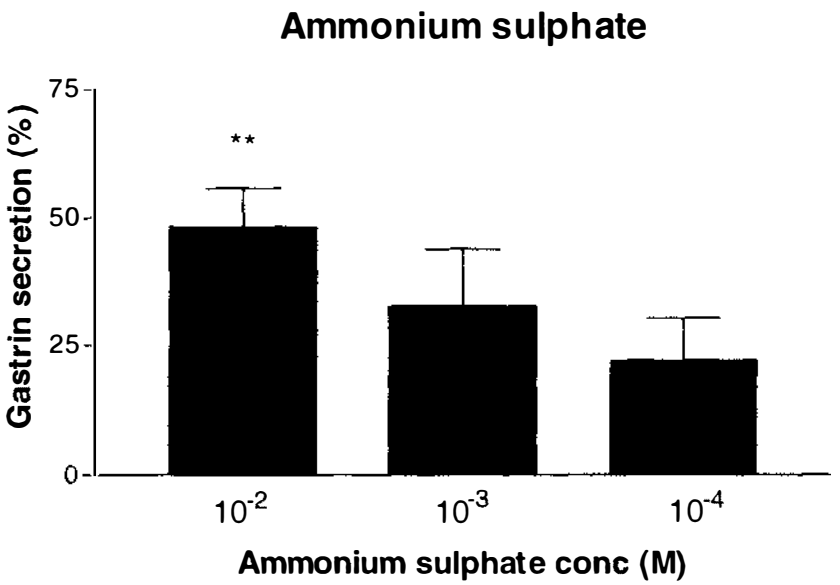

Figure 6.2. Effect of ammonium sulphate from 10^{-4}M to 10^{-2}M on *in vitro* gastrin secretion. Bars show mean±SEM, **=p<0.01, results different from control.

were not adjusted for osmotic pressure. When solutions were adjusted to 320-330mOsmL^{-1}, basal media with 2% acetic acid produced a 36% stimulation ($p<0.05$) of gastrin secretion.

6.3.3.2 Salts

Neither NaCl nor Na_2SO_4 had any effect on basal gastrin secretion (0±10% and -8±10%) when solutions were adjusted to 320-340mOsmL^{-1}. Ammonium sulphate produced a dose dependent increase in gastrin secretion (Figure 6.2). The graph shows the lower doses of ammonium sulphate tested, where the amount of salt acid did not result in solutions with excessive osmotic pressures (not exceeding 360mOsm.L^{-1}). Ammonium sulphate at concentrations of 0.05M, 0.1M and 0.2M were also tested, and these produced results not significantly different from control values. These solutions had osmotic pressures of over 400mOsm.L^{-1} however, which may be the cause of decreased gastrin secretion in these solutions compared to solutions containing ammonium sulphate at lower concentrations.

6.3.3.3 Detergents

Tween 80 at all concentrations but 0.001% caused increased gastrin release in the *in vitro* tissue test. At 0.05% levels stimulation ($p<0.05$) was 175±13% and greater than this at higher concentrations. Nonidet had caused 52±15% stimulation ($p<0.05$) of gastrin release from 0.05% upwards. Triton X100 caused 85±26% stimulation ($p<0.05$) at 0.001% and greater than 100% at all other concentrations. Thesit did not affect basal gastrin secretions (20±11%, $p>0.05$), but had no effect on ultramembrane filtration of RI-9/RI-12. In the experiment where it was used, there was no appreciable loss of activity from retentate solutions in the filtrations conducted without Thesit nor in the filtrations conducted with Thesit.

6.3.3.4 Hydrophobic interaction chromatography

There was no effect on *in vitro* gastrin secretion in fractions of the blank run containing elution buffer only. Freeze drying did not remove the tissue gastrin inhibitory effects from rumen incubate solutions.

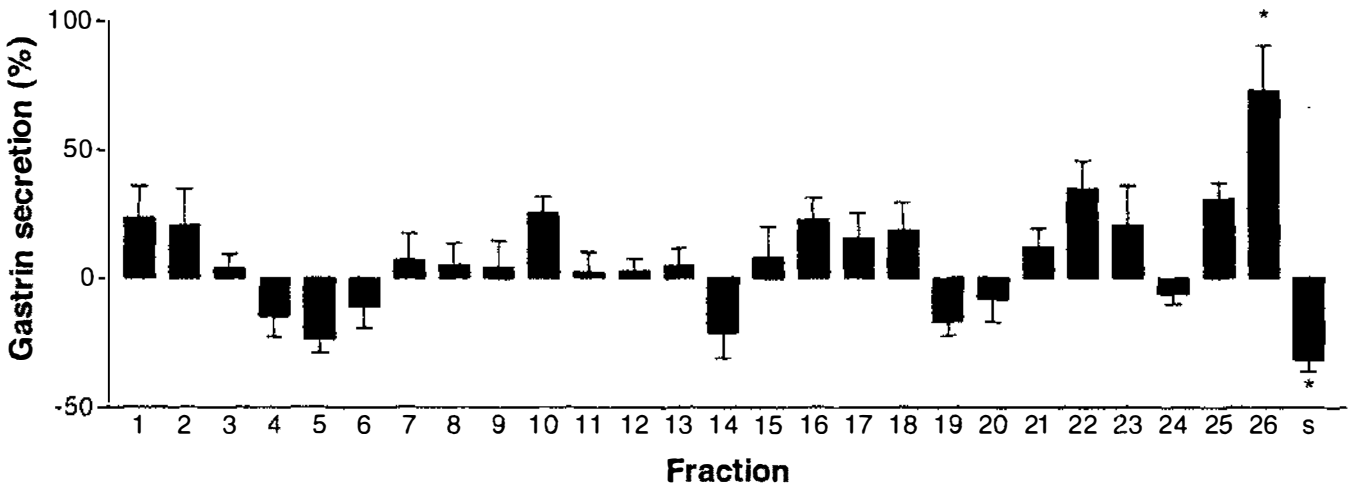

Figure 6.3. Fractions from a Hydrophobic interaction chromatography column to which 4mL of 60% concentrated rumen incubate with inhibitory activity was added. Shown are the mean±SEM of *in vitro* gastrin response to fraction, S= the sample prior to its being added to the column, which was significantly different to control gastrin secretion, $p<0.05$, result different to control.

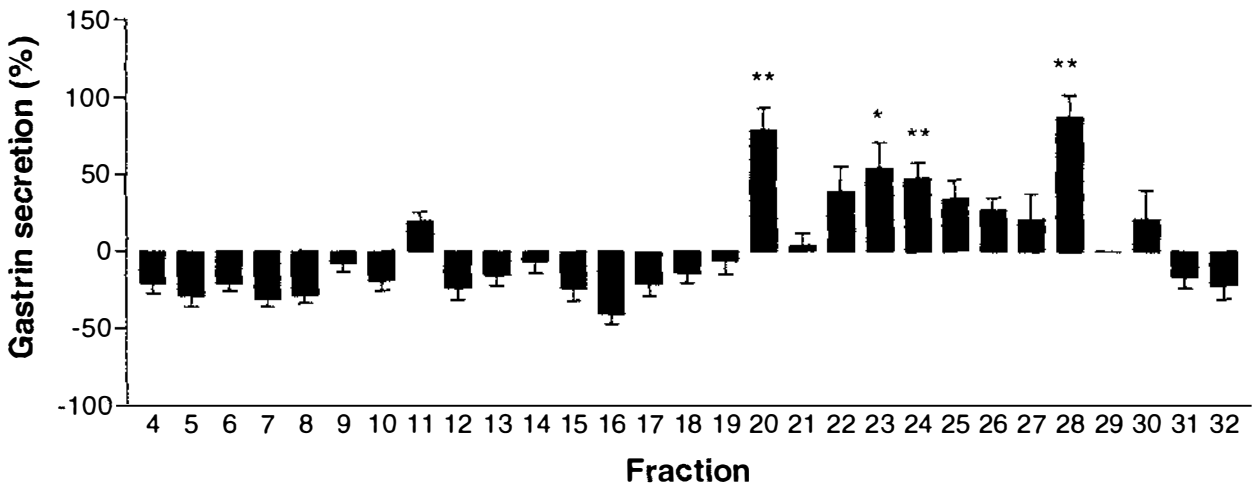

Figure 6.4A. Fractionation of 20mL of a rumen incubate (RI-23) on a Hydrophobic interaction column. Shown are the mean±SEM of *in vitro* gastrin response to fraction, *=p<0.05, **=p<0.01, secretion different from control.

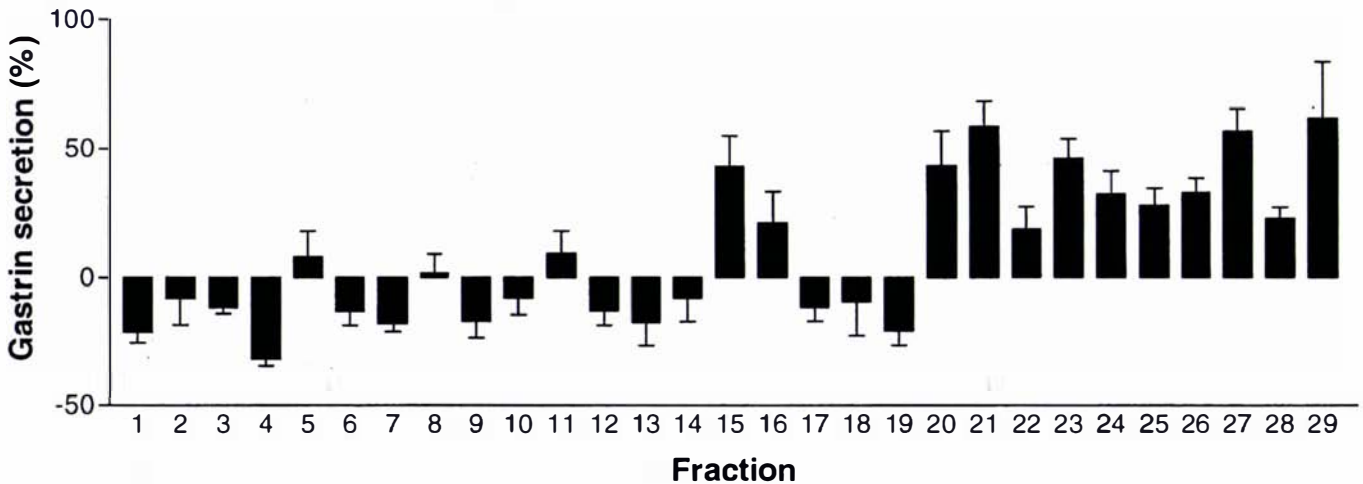

Figure 6.4B. Fractionation of 20mL of a rumen sample (RUM-26) on a Hydrophobic interaction column. Shown are the mean±SEM of the *in vitro* gastrin response to fraction.

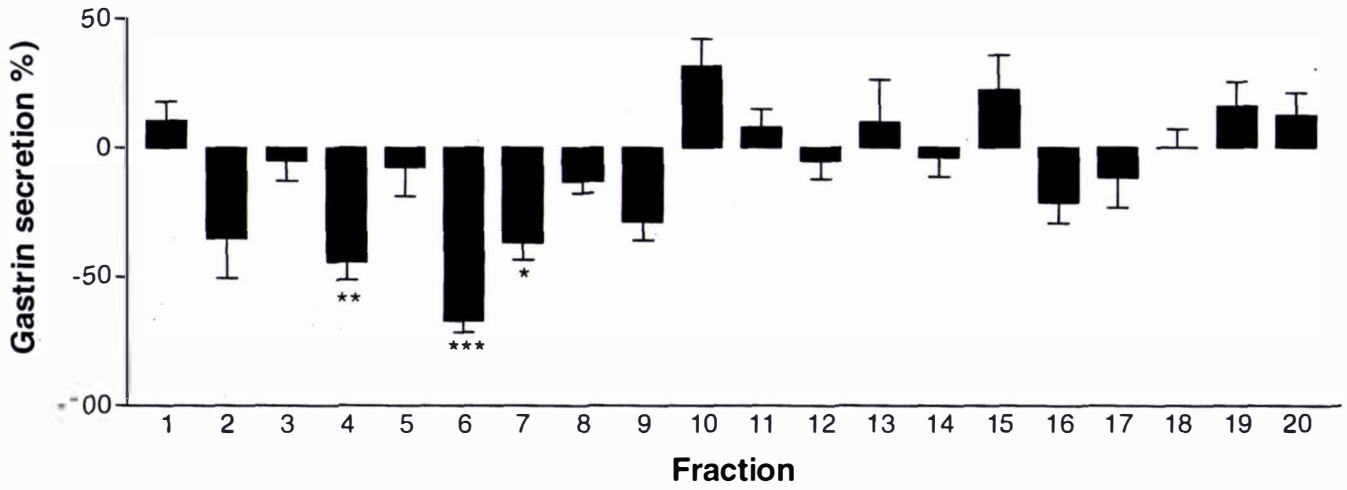

Figure 6.4C. Fractionation of 50mL of an abomasal incubate (AI-8) on Hydrophobic interaction column. Shown are the mean±SEM of the *in vitro* gastrin response to fraction, *=p<0.05, **=p<0.01, ***=p<0.001, secretion different from control.

Fractionation of a 4mL freeze dried sample of RI-22 produced fractions which caused slight decreases in gastrin secretion in three areas, but effects were not statistically significant (Figure. 6.3). The three areas relate to different stages of elution, with fractions 4, 5 and 6 eluting under conditions of high salt concentration, fraction 14 eluting in moderate salt concentrations and fractions 19 and 20 eluting at low salt concentration.

Fractionation of 20mL of RI-23 (Figure6.4A) and RUM-26 (Figure 6.4B) produced similar patterns of activity. Tissue responses to the column fractions were variable in both cases, which meant that none of the fractions produced caused statistically significant gastrin responses. Small decreases in gastrin secretion caused by fractions eluted under high salt concentration. Stimulation of gastrin release occurs with rumen incubate fractions which eluted at low salt concentrations.

Fractionation of 50mL of AI-8 did produce fractions which inhibited gastrin release under conditions of high salt concentration (Figure 6.4C). For this set of results, the first three fractions are the runoff from loading the column. They did contain significant amounts of phenol red, showing that constituents of the samples did run off the column during loading. The lack of inhibitory activity in these samples suggests that activity is being held at least partially by the column. The last 10 fractions of this column were not assayed due to low supply of gastrin antiserum.

6.4 Discussion

Crude separation of activity was achieved with the ultramembrane filtration of rumen fluid and rumen incubate samples. However, the ultramembrane filtrations of rumen fluid samples and rumen incubates had different characteristics. Tissue gastrin inhibitory activity did not pass through any of the ultrafiltration membranes with rumen fluids or rumen incubates, but a stimulant of gastrin secretion was present in the filtrates of both. In general, for the rumen fluid samples, tissue gastrin inhibitory activity was present in the retentate. For the rumen incubates, in general, activity was lost during filtration appearing in neither the filtrate nor the retentate, and could not be washed off

the filter membrane. It is suggested that the activity binds very strongly to the ultramembrane filter with rumen incubates. These results suggest that the activity present in rumen fluids and rumen incubates is different. The filtration properties of rumen fluids and rumen incubates did not change with the addition of acetic acid, NaCl, glycerol, or alcohol.

Binding of activity to membranes in the case of rumen incubates and aggregation with rumen fluids meant that the molecular weight of the inhibitory activities could not be determined by ultramembrane filtration. Both aggregation and adherence to filters suggest that separation of the inhibitory compounds, in rumen fluids and rumen incubates, using standard gel filtration or ion exchange chromatography may result in the loss of activity. Further, the tissue test system was not tolerant to the use of detergents. Thus, any such compounds must be removed before activity can be tested with the *in vitro* tissue system. Dialysis would normally be used to remove or dilute such chemicals, but the activity being isolated shows a tendency to adhere to dialysis tubing. The use of hydrophobic interaction chromatography using a neutral salt solution, which is more suitable for separation of hydrophobic compounds may be of more use. Even hydrophobic interaction chromatography has produced poor yields.

6.4.1 Effects of detergents, salts and solvents on gastrin secretion

The effects of various ions and solvents on *in vitro* gastrin secretion were tested. The aim of these tests was to find salts or solvents that would keep the tissue gastrin inhibitory activity in solution during the filtration of both rumen fluids and rumen incubates. Of the solvents tested, alcohol and glycerol were found not to affect tissue gastrin secretion. Of the salts, NaCl and Na_2SO_4 did not affect basal gastrin secretion from tissue. Alcohol was thought unsuitable for filtrations, as the alcohol may evaporate during the filtration. Neither glycerol nor NaCl when added to test solutions resulted in tissue gastrin inhibitory activity appearing in filtrates. Despite this, the effects of salt and solvents on

basal gastrin secretion provide information on the control of gastrin secretion, which is of interest.

Results presented here show that alcohol itself does not stimulate gastrin release from ovine antral tissue. This is in general agreement with results in humans where ethanol may (Singer *et al.,* 1987; Chari *et al.,* 1993) or may not (Peterson *et al.,* 1986b; Singer *et al.,* 1991) stimulate acid release, but does not stimulate gastrin secretion (Peterson e*t al.,* 1986b; Singer *et al.,* 1987; Chari *et al.,* 1993).

In humans solutions containing fats have been shown to stimulate gastrin release slightly (Richardson *et al.,* 1976), but intragastric fat solutions are unable to stimulate somatostatin increases (Schudziarra *et al.,* 1978). Thus, it was expected glycerol, a component of fats, would have little effect on gastrin or somatostatin secretion *in vitro*.

As a factor in separation chemistry, acetic acid was an undesirable solvent, because of its osmotic effect and also because of the possibility that it stimulates gastrin secretion. One experiment suggested that acetate has a stimulatory effect on gastrin secretion. Acetate may well be a factor present in rumen solutions that stimulates gastrin release. Acetate often forms a major proportion of the volatile fatty acids present in the rumen, which can be between 60-120mM (Stevens and Hume, 1998). In addition acetate is absorbed more slowly than propionate and butyrate (Stevens and Hume, 1998), so may be present in significant amounts in the abomasum. As an important part of the ruminant diet, organic acids may well stimulate gastrin release in ruminants. Butyrate and acetate infused into sheep did not affect gastrin secretion (H. Simpson, unpublished results). This suggests the actions of acetate are likely to be luminal.

The inclusion of the nonionic detergents Triton X100, Tween 80 and Nonidet all resulted in increased gastrin levels in a dose dependent fashion. This too is thought to be a non-receptor effect and is probably due to G cell membrane damage resulting in the general release of gastrin into the incubation medium. Detergents such as Triton X100 are routinely used to dissolve and dissociate membrane proteins, so it seems likely that the detergents have this effect on G cell membranes also. It is possible to remove detergents by dialysis, but as the G cell is sensitive to very small amounts of

detergent in solution (0.001% of solution and greater), it is undesirable to include detergents in test solutions.

6.4.1.1 Effects of ammonia on gastrin secretion

In terms of separation experiments, the stimulatory action of ammonia on the G cell makes it unsuitable as a buffer salt. However, the effects of ammonia on gastrin secretion may be particularly relevant to abomasal parasitism, as recently, *O. circumcincta* has been shown to produce large amounts of ammonia *in vitro* (Scott, Merkelbach, Simpson, unpublished results). When ammonium sulphate, but not sodium sulphate, ions are added to *in vitro* solutions they stimulate gastrin secretion. However, this is not the stimulant present in the rumen incubates, as levels of ammonia in these solutions were measured (Merkelbach, Scott and Simpson, unpublished results) and were low.

Elevated ammonia levels may produce hypergastrinaemia in humans infected with *H. pylori*. Increased luminal ammonia is a feature of *H. pylori* infection (Yoshida *et al.,* 1989; Chittajalu *et al.,* 1991a, b), though its effects on gastrin secretion are debated. Short term alterations of ammonia levels in the human stomach do not affect gastrin secretion (Chittajallu *et al.,* 1991a, b; El Nujumi *et al.,* 1991; Graham *et al.,* 1991). However, increased ammonia in the diet over the long term in rats caused increased gastrin secretion and production along with increased mucosal thickness, possibly due to nonspecific tissue injury (Litchtenberger *et al.,* 1995). In addition, ammonia solution applied to gastric mucosa for two to four weeks in rats decreased the number of D cells, which may be the cause of hypergastrinaemia in response to ammonia (Iyo *et al.,* 1999). Thus, there is a precedent that ammonia does increase gastrin secretion, though there is no evidence of this in short term tissue cultures. However, ammonia is released in higher concentrations by *Ostertagia* worms (Merkelbach, Scott, Simpson, unpublished results). Thus it is possible ammonia is responsible for hypergastrinaemia in abomasal nematode infections. During such infections the abomasum is in an inflamed state (see Chapter 3) and the inflamed mucosa is more sensitive to the stimulatory effects of ammonia (Dial *et al.,* 1996).

In addition ammonia may alter the production and secretion of gastrin, in a similar fashion to dietary amines. The dietary amines tryptamine and tyramine inhibit G34 cleavage to G17 probably by passively permeating the vesicular membrane and raising vesicle pH as efficacy is related to hydrophobicity (Hussain *et al.,* 1999). While most amines will not exist in the basic form in the low pH of the stomach, they will become more so as gastric pH rises and are a possible mechanism by which food contents in the gastric lumen may modulate not only gastrin secretion, but the profile of gastrins present in the general circulation (Hussain *et al.,* 1999).

6.4.2 Hydrophobic interaction chromatography

Hydrophobic interaction chromatography was employed in an attempt to purify the active inhibitory compounds in incubate solutions. The success of this method was limited by the low yield of inhibitory activity in the eluting fractions. The fractions added to the column were from rumen fluid, rumen incubates and an abomasal incubate, all with very little “breakdown” activity. All samples fractionated revealed the same pattern of activity in the fractions. This suggests that the activity in the samples was very similar.

The yield of active product off the hydrophobic interaction column was low. This necessitated either freeze drying samples or adding large volumes to retrieve activity in fractions. When 4mL was applied to the column, which contained enough activity to produce an inhibitory effect in one tissue assay plate, no significant activity was yielded in fractions. In fact, the only statistically significant responses were produced with a 50mL fraction added to the column. It was thought possible that activity was being diluted during the elution and thus activity was not being detected.

Activity that does elute from the column tends to come off at high salt concentrations, suggesting it is not binding well to the column. There does appear to possibly be a fraction in the middle of the elution that may have bound to the column, but recovery levels are small and never have significant effects on gastrin secretion. Activity eluting from the column early in fractionation suggests that the compound responsible is hydrophilic. This contradicts results from the ultramembrane filtrations, which suggest that the

gastrin secretion inhibitory compound is hydrophobic. It is possible that this activity is “breakdown” activity, which has been concentrated on the column from the large volumes of sample added. It was not possible to test these fractions for “breakdown” activity as they were of high salt concentration which could affect gastrin and antibody binding, and could compromise results. This seems unlikely however, as activity was present at the start of the elution in all fractions, even the freeze RI-23, which was only concentrated threefold by freeze drying. It is also possible that the secretion inhibitory compound bound well to the column and eluted in several of the later fractions, but its effects were masked by the stimulatory activity present. Whatever the binding characteristics of the secretion inhibitory activity to the column were, separation of the activity using hydrophobic interactions chromatography yielded poor results.

6.4.3 Ultramembrane filtrations

The ultramembrane filtrations of the rumen fluids and rumen incubates yielded the most information about the activity present in these solutions. Both rumen fluids and rumen incubates contained a gastrin secretion stimulant. This stimulant was of low molecular weight and could pass through all of the ultramembrane filters.

In general, inhibitory activity in the rumen solutions and rumen incubate solutions could not pass through any of the ultramembrane filtration membranes. There were exceptions, the activity in RUM-33 passed through YM10, YM30 and XM50 membranes, and RUM-29 passed through YM30, possibly due to incorrect filtering. Some activity from RUM-32 may also have passed through YM10 and XM50 though the effects in filtrate were not significant. Activity appearing at greater levels in retentate than observed in the original solution is likely to be due to a concentration of the compound responsible and removal of competing stimulants during ultramembrane filtration. Aggregation of active compounds forming a precipitate may be responsible for activity not passing through filters.

For rumen incubates in general, all inhibitory activity was lost during ultramembrane filtration, presumably bound to the membrane (Tables 6.5, 6.6,

6.7 and 6.9). The exception to this was the composite sample RI-9/RI-12 which did have activity in the retentate after ultramembrane filtration. This may have been a result of repeated freezing and thawing of sample. In the majority of samples tested, tissue gastrin inhibitory activity showed different separation properties between rumen fluids and rumen incubates. This suggests that the activity present in rumen fluids and rumen incubates is different.

Rumen solutions were shown in experiments in Chapter 4 to have significant gastrin "breakdown" activity which was minimal or absent in rumen and abomasal incubates. It seems likely that this is the activity present in the rumen fluid retentates. "Breakdown" activity was not tested in the rumen fractions passed through ultramembranes, because the presence of such activity in rumen fluids was not realised at the time. Repeated freezing and thawing also produced activity in the retentate of ultramembrane filtrations of incubates. This is further evidence that "breakdown" was the activity remaining in retentates, as gastrin "breakdown" activity is increased in rumen incubate samples which are repeatedly frozen and thawed (Table 5.4B).

The activity lost by rumen incubates during ultramembrane filtration seems likely to be the secretion inhibitor, which is the prominent activity in rumen incubate solutions. Any secretion inhibitor present in the rumen solutions is likely to adhere to the ultrafiltration membrane also, but this effect is masked by the presence of "breakdown" activity in the retentate. This is supported by the loss of inhibitory activity during the filtration of RUM-27 and RUM-31 through YM3 filters (Table 6.1). Although activity is present in the retentate of YM10 and YM30 membrane filtrations of RUM-29 and RUM-31 (Table 6.8), this is likely to be due to filtration being more complete through these membranes, concentrating "breakdown" activity in retentate and removing the gastrin secretion stimulant, which appears in the filtrate of these samples (Tables 6.1 to 6.4).

6.4.4 Alternative separation methods

The next steps in isolating this compound are establishing the molecular weight and chemical composition and if it is shown to be a protein or peptide, the amino acid sequence. Once these are known, it would be possible to

speculate the actual source of the active molecule, screen for its presence in various solutions and determine under what conditions it may be produced.

6.5 SUMMARY

The *in vitro* gastrin inhibitory activity in rumen fluids and rumen contents showed different characteristics, but neither passed through ultramembrane filtration. The filtrates of both incubates and rumen fluids contained a stimulant of gastrin secretion. In the case of rumen contents, activity was present in the filtration both in solution and in an aggregate formed by filtration. For the rumen incubates, gastrin inhibitory activity was generally lost during filtration. Inhibitory activity could not be removed from ultrafiltration membranes by solvents after filtration.

Activity appeared to be hydrophobic in nature, but the tissue *in vitro* test was damaged by the present of ionic detergents, which precluded their use. Hydrophobic interaction chromatography was used to separate activity, but in general had low yield and was of limited success. From all the results obtained, it is difficult to say what exactly the active molecule is. Smaller hydrophilic compounds can be eliminated as they should pass through the membrane. A lipid compound would not be expected to be labile to alterations in pH and temperature and the *in vitro* inhibitory activity has proved to be so. A protein with tertiary structure is possible as acid disrupts tertiary structures in proteins. Proteins are also temperature sensitive. A peptide is also a likely candidate, though these tend to be a little more robust than proteins as they have less rigid structure. Peptides are typical effector molecules for endocrine cells.

CHAPTER 7

GENERAL DISCUSSION

The synthesis, secretion and physiological effects of amidated gastrins have been extensively studied in monogastric animals, as has the hypergastrinaemia associated with human gastric diseases. In contrast, the control of gastrin secretion in ruminants and the causes and effects of hypergastrinaemia during abomasal parasitism in sheep are still unclear.

Hypergastrinaemia has been suggested to have both beneficial and detrimental effects to the host animals during abomasal parasitism. The proposed beneficial effect is promoting parietal cell recovery (Scott *et al.,* 1998b), which in turn would restore acid secretion and increase digestive efficiency. The reported detrimental effect is the reduction in food intake (Fox *et al.,* 1989a, 1997), which usually results in weight loss and reduced liveweight gain.

In cattle, the hypergastrinaemia associated with abomasal infection can be explained almost entirely by abomasal hypoacidity (Fox *et al.,* 1993), however, in sheep, the relationship between hypergastrinaemia and abomasal hypoacidity is not as strong. Lawton *et al.* (1996) showed that in *O. circumcincta*-infected sheep, whereas hypoacidity initially increased at the same time as gastrin secretion, serum gastrin levels often remained elevated after abomasal pH returned to normal. Furthermore, in some sheep, hypergastrinaemia was abruptly reversed when abomasal pH increased over 5.5. This feature had not been reported previously, and was suggested to be due to the action of microbial products. This was supported by the presence of a potent inhibitor of *in vitro* gastrin secretion in cultures of abomasal microbes. The work presented here therefore addressed several questions: how gastrin secretion is controlled; what are the effects of hypergastrinaemia during abomasal parasitism; whether microbial products present in the abomasum can affect the hypergastrinaemia observed during parasitism.

What are the key features of the control of gastrin secretion in unparasitised ruminants?

Although the cephalic control of gastrin secretion has been shown to exist, the more constant flow of digesta into the abomasum, when compared with monogastric animals, suggests that luminal chemicals may be important in the control of gastrin secretion in ruminants.

At the level of the G cell, the principal stimulatory and inhibitory pathways controlling gastrin secretion in sheep appear to be similar to those in monogastric animals. *In vitro* preparations of ovine antral segments, first developed by Lawton (1995) and used in the present experiments, showed that the principal stimulant of gastrin is GRP, with cholinergic pathways also present. Adrenaline was ineffective in these experiments and those of Lawton (1995), thus there appears to be no adrenergic input to the G cell in sheep. The present studies also showed that somatostatin was able to reduce stimulated, but not basal, gastrin secretion. VIP was also able to inhibit stimulated gastrin secretion, most likely via increased release of somatostatin. The ineffectiveness of somatostatin on basal gastrin has been suggested to be due to maximal restraint of gastrin secretion by somatostatin in the *in vitro* preparation used (Lawton, 1995). A high basal level of restraint on gastrin by somatostatin is also reported in sheep *in vivo* (Shulkes *et al.,* 1994; Zavros *et al.,* 1999).

Chemicals likely to be present in abomasal fluid, namely aromatic amino acids, ammonia and acetate, are capable of increasing gastrin secretion *in vitro* (Chapters 2 and 6). These may be the low molecular weight (less than 3000M_r), acid-resistant stimulants of gastrin secretion in rumen fluid. Alternatively, rumen fluid may contain gastrin stimulants similar to those produced during the fermentation processes used to make alcoholic beverages (Petersen *et al.,* 1986b; Singer *et al.,* 1987; Hajnal *et al.,* 1988; Teyssen *et al.,* 1997), which are thermostable, anionic, polar substances of molecular weight less than 700 Daltons (Teyssen *et al.,* 1997, 1999), suggested to be glucose metabolites (Singer *et al.,* 1991; Teyssen *et al.,* 1991). Other candidates for the stimulatory activity in abomasal and rumen contents are: magnesium ions (Peterson *et al.,*

1986a), calcium ions (Ray *et al.,* 1997; Seensalu *et al.,* 1997), amines (Lichtenberger *et al.,* 1982a, b; Dial *et al.,* 1986, 1991; DelValle and Yamada, 1990) and L amino acids (Konturek *et al.,* 1977a; Lichtenberger *et al.*, 1982a, b; Dial *et al.,* 1986, 1991; DelValle *et al.,* 1990).

The role of gastric acidity in the control of gastrin secretion in the unparasitised sheep is unclear, as there was no significant correlation between serum gastrin and abomasal pH. Continuous measurement of abomasal pH in sheep has shown that it does fluctuate in the unparasitised animal (Hertzberg *et al*., 2000) over a similar range to that reported here. It therefore appears that abomasal pH has little effect on gastrin secretion over the small range pH fluctuates in unparasitised sheep. However, marked increases in abomasal pH, induced either by abomasal parasitism (Lawton *et al.,* 1996) or bicarbonate infusion (Reynolds *et al.,* 1991), do increase gastrin secretion. Thus, it seems likely that acidity and cephalic pathways have only minor effects on gastrin secretion in the unparasitised ruminant, so luminal constituents may play a significant role in stimulating gastrin secretion.

What are the effects of hypergastrinaemia during abomasal parasitism and what causes the increased secretion of gastrin?

Hypergastrinaemia may have the detrimental effect during parasitism of inducing anorexia. The best evidence for this action is the close correlation between increased serum gastrin levels and reduced food intake in *O. ostertagi*-infected calves (Fox *et al.,* 1993) and calves treated with omeprazole (Fox *et al*, 1989a; Fox, 1997). In contrast, in sheep, reduced food intake did not correlate well with either increased serum gastrin concentrations or abomasal pH (Chapter 3). In one animal (Sheep 5, LPI-1, Chapter 3), hypergastrinaemia was present but food intake was not reduced and, conversely, food intake was reduced in animals at times when serum gastrin levels were within normal ranges (Chapter 3). The anorexia may be a result of gastric pain or inflammation of the mucosa. The inflammatory mediator TNFα has been shown to suppress food intake (Holden and Pakula, 1996), and is a candidate for the mediation of reduced food intake during a parasite infection.

Scott *et al.* (1998b) suggested that hypergastrinaemia could both promote the regeneration of the depleted parietal cell population resulting from *O. circumcincta* infection as well as stimulate secretion from surviving parietal cells. Mucosal hyperplasia is a common feature of abomasal parasite infection, and has been attributed to hypergastrinaemia (Anderson *et al.,* 1988). Experiments in transgenic mice have shown that gastrin is primarily involved in the differentiation and maturation of cells in the gastric gland, in particular the parietal cells and ECL cells, rather than in their proliferation (Wang and Dockray, 1999). In transgenic mice in which the gastrin gene has been deleted, the total absence of gastrin caused a reduction in parietal cell numbers with those present producing little or no acid (Koh *et al.,* 1997; Friis-Hansen *et al.*, 1998). Hypergastrinaemia in the present studies did not appear to promote the regeneration of parietal cells (Chapter 3), as both parietal cell numbers and distributions within the glands were no different in the presence or absence of hypergastrinaemia. These results do not necessarily negate a role for gastrin in the maintenance of parietal cell differentiation. Instead, they suggest that only basal levels of serum gastrin may be necessary for the maintenance of the parietal cell population in the fundus and elevation of serum gastrin has no further benefit.

The present experiments highlight the role of inflammation and tissue damage in affecting gastrin secretion. This was apparent from the differences seen when sheep were infected by adult transplant or with larvae which infect either the antrum of the fundus. During fundic infection, particularly with transplant of adult parasites (API group, Chapter 4) into the abomasum, the relationship between serum gastrin and abomasal pH was close, but when larval development was predominantly in the antrum (LPI-1 group, Chapter 3), the relationship between abomasal pH and serum gastrin was poor. The mucosal damage caused by larval development in the antrum reduced the number of G cells. Mucosal damage has also been suggested to explain the decrease in gastrin content of the antrum in *H. pylori*-infected humans with severe gastritis (Sankey *et al.*, 1990). Thus, depletion of tissue gastrin may be responsible for the unexpectedly low serum gastrin concentrations in animals with elevated abomasal pH. Both Purewal *et al*. (1997) and Scott *et al*. (1998a) have shown that tissue gastrin levels decrease during *Ostertagia* infection,

despite increased gastrin production (Purewal *et al.*, 1997). Depletion of tissue gastrin may be the cause of the reduction in serum gastrin levels at high abomasal pH in the two animals in which such effects were observed present study (197 and 210, LTI group, Chapter 4), and of the reversal of hypergastrinaemia reported by Lawton e*t al*. (1996).

Although tissue damage may reduce serum gastrin concentrations, inflammation may augment the serum gastrin response to abomasal hypoacidity. Serum gastrin concentrations during *O. circumcincta* infection often exceed those produced by direct infusion of rumen fluid or bicarbonate into the abomasa of sheep (Reynolds *et al.,* 1991). The inflammation commonly associated with abomasal parasitism (Armour *et al.,* 1966; Klesius, 1993; Stear *et al.,* 1996; Scott *et al.,* 2000) may be responsible for this. Inflammatory mediators have been shown to stimulate gastrin release in several species (Lehmann *et al.,* 1996; Weigert *et al.*, 1996; Beales *et al.*, 1997). Further, the *in vitro* pharmacology experiments (Chapter 2) suggested that the G cell had increased sensitivity when tissue was obtained from parasitised abomasa, in common with results obtained in humans (Graham *et al.,* 1991; Beardshall *et al.,* 1992; Annibale *et al.,* 1996; Gibbons *et al.,* 1997). Inflammation may also be responsible for the persistence of hypergastrinaemia after abomasal pH returns to normal levels (Lawton *et al.*, 1996; Chapters 3 and 4).

Does a microbial inhibitor affect gastrin secretion *in vivo*?

Lawton *et al.* (1996) first proposed that increased microbial numbers in hypoacidic abomasal fluid may inhibit gastrin secretion, explaining the sudden reversal of hypergastrinaemia at high abomasal pH in some animals. Further, this hypothesis was supported by the production of an inhibitor of *in vitro* gastrin secretion by abomasal microbial incubates. In the present studies, anaerobic bacterial numbers did increase rapidly with only small elevations in abomasal pH and, in addition, an inhibitor of *in vitro* gastrin secretion was present in abomasal contents when pH increased to over 4.5. However, neither increased bacterial numbers, nor the presence of an inhibitor of *in vitro* gastrin secretion, was consistently associated with abrupt decreases in serum gastrin

concentrations in parasitised sheep. Therefore, the hypothesis that serum gastrin may be affected by microbes at high abomasal pH was not supported by the present study (Chapter 4).

In many animals infected with *O. circumcincta*, disparities existed in the expected relationship between abomasal pH and serum gastrin, but in most cases the action of a microbial inhibitor affecting gastrin secretion could be discounted. *In vitro* gastrin inhibitory activity was always present in abomasal fluid when the pH of the sample was 4.8 or greater, and in some cases was also present at lower pH levels, but there was no consistent evidence that *in vitro* inhibitory activity reduced serum gastrin concentrations. Lawton *et al.* (1996) suggested that the bacterial inhibition of gastrin secretion occurred when pH increased to 5.5 and above, however, elevation of abomasal pH to as high as 7.0 did not reduce serum gastrin levels in most cases (Chapter 4).

The inhibitor of *in vitro* gastrin secretion may still affect gastrin secretion *in vivo* under specific conditions. It may be necessary for bacteria to be present in antral glands for an inhibition of gastrin secretion to occur. Bacterial invasion of the fundic pit, but not the antral gland, has been reported in calves with diarrhoea and ill-thrift (Gunning, 2000). Although there are no reports of bacterial invasion of the antral gland during abomasal infection in sheep, it is possible that it may cause the reversal of hypergastrinaemia reported by Lawton *et al*. (1996).

Alternatively, antral inflammation may be necessary to increase the permeability of the antral mucosa to allow inhibitory factors access to G cells. If this were the case, inhibition of gastrin secretion by microbial products should have occurred in the LPI-1 group (Chapter 3), which were infected with the strain of *O. circumcincta* larvae which predominantly infected the antrum. Although elevation of serum did lag behind elevation of abomasal pH in these animals, this occurred only during early infection in most animals and was not pH-dependent.

What is the source of the gastrin inhibitory activity in hypoacidic abomasal contents?

The inhibitory activity in hypoacidic abomasal contents is likely to be a microbial product, as it can be generated by cultures of both abomasal and rumen fluids. The actual bacterial species surviving in the abomasum, and those producing the gastrin inhibitory activity, were not examined. In some abomasal incubates containing inhibitory activity, the dominant species present in solution were members of the Actinomycete family. This suggests that the inhibitory activity is caused by a product common to many bacterial species, being present in rumen fluid, which contains mostly obligate anaerobic bacteria (Hungate, 1966; Leedle and Hespell, 1980), in aerobic cultures of abomasal, and rumen fluids and also in *S. bovis* monocultures. A large bacterial population at, or close to, the maximum number supported by the medium was present in all solutions containing inhibitory activity. The inhibitory product may be released by bacterial breakdown, as activity can in some cases be increased by repeated freezing and thawing, and the it is present only with large bacterial populations.

The rumen is probably the origin of the inhibitory activity in the parasitised abomasum. Rumen fluid contained three factors which affected gastrin concentration in the *in vitro* tissue test system: an inhibitor of secretion; a stimulant of secretion and a factor which removed gastrin from solution, referred to as "breakdown". Inhibitory activity is probably always present in rumen fluid, but its effect is often masked by the "breakdown" effect, making it impossible to demonstrate the presence of an inhibitor of gastrin secretion. The inhibitor of gastrin secretion becomes apparent in rumen fluid when "breakdown" activity is lower than that in tissue tests. It is proposed that, generally, when rumen fluid passes into the abomasum, both the "breakdown" effect and the inhibitor of gastrin secretion are inactivated by the acidic conditions. This is supported by the sensitivity of both to acidity when tested *in vitro* (Chapter 5). However, as conditions in the abomasum become less acidic during parasitism, the inhibitory activity survives (Chapter 4). One unexplained observation was the general lack of "breakdown" activity in the hypoacidic abomasum, despite its prominence in rumen fluid. The "breakdown" activity

may be more sensitive to acidity than the inhibitor, as shown in the one incubate where this was tested. The “breakdown” activity could also be more sensitive to degradation by pepsin in the stomach. However, neither sensitivity to acidity or pepsin could explain why little to no “breakdown” activity was observed in abomasal solutions with pH as high as 6.7.

Bacterial proliferation is less likely than survival of rumen bacteria. Contrary to observations in previous studies (Nicholls *et al.,* 1987), bacterial numbers increased very rapidly with only very small elevations in abomasal pH, being near maximal at pH3.5 and above. The model relating abomasal pH and the effect of abomasal fluid on *in vitro* gastrin secretion showed that inhibitory activity also began to appear at pH3.5, had maximal effects at pH5.3 and over, with responses being statistically significant at pH4.8 and over. The marked increase in bacterial numbers with such small changes in abomasal pH suggests that survival of rumen bacteria, not increased proliferation is responsible. Even at pH 4.5, when significant amounts of inhibitory activity were present in the abomasal contents, bacterial metabolism and growth are likely to be minimal, assuming the bacteria present originated in the rumen. Rumen pH generally varies between 5 and 7, though pH6 and below is considered low (Gentile *et al*., 1999). Decreased pH is associated with decreased fermentation and reduced cellulose breakdown by rumen bacteria (Argyle and Baldwin, 1986; Hoover *et al*., 1988). This suggests that fermentation is likely to be minimal in conditions in which inhibitory activity first becomes significant in the abomasum, and that the inhibitor of gastrin secretion is a rumen product which survives in the abomasum.

What is the inhibitor of gastrin secretion likely to be?

The bacterial inhibitor of gastrin secretion was pH and temperature sensitive and appeared to be very hydrophobic. These properties suggest that it is a protein or polypeptide. If the inhibitory compound is a peptide or protein, its hydrophobicity may be due to its peptide sequence containing mostly hydrophobic amino acids, or it being folded in a hydrophobic conformation, or that hydrophobic sidechains are present. The hydrophobicity made separation difficult, but the biggest constraint was that the method of testing for activity involved tissue had little tolerance to detergents and hydrophobic solvents.

The exact identity of the factor which inhibits gastrin secretion is unknown. There are some reports of gastrin inhibitors produced in the diet of other species, but it seems unlikely that the inhibitor in the present experiments is related to these compounds.

The only other report of fermentation products reducing gastrin secretion comes from studies in rats fed a diet of indigestible carbohydrates (Gee *et al.*, 1996). These authors suggested that a fermentation product from the colon was responsible for increased blood enteroglucagon and decreased gastrin levels. The properties of this factor were not identified, and it would have to reach the G cell via the circulation, unlike the product in the present experiments.

The inhibitor could be a bioactive peptide, similar to caseinomacropeptide or glycomacropeptide from milk. Glycomacropeptide, and peptides formed from its hydrolysis, inhibited acid (Vasilevskaia *et al.,* 1977; Stan and Chernikov, 1979; Aleinik *et al.,* 1986) and gastrin secretion (Stan and Chernikov, 1979; Aleinik *et al.,* 1986; Stan *et al.,* 1986). The gastrin inhibitor present in caseinomacropeptide is sensitive to acidity (Scanff *et al.,* 1992), as is the secretion inhibitor reported in the present study. Evidence against the inhibitor being similar to the caseinomacropeptide inhibitors are the differing separation properties. The active fractions of caseinomacropeptide are easily separated by gel chromatography (Chernikov *et al.,* 1979), suggesting they are not as hydrophobic as the inhibitory factor in the present experiments.

If the inhibitory factor were acting via a physiological mechanism, it would most likely be either a compound resembling somatostatin or one which stimulates somatostatin release. It was not possible to measure the effects of either rumen fluids or rumen incubates on somatostatin secretion, as both caused interference in the somatostatin assay. However, the activity of the bacterial gastrin inhibitor differed from that of somatostatin as, unlike that peptide, the inhibitor was able to inhibit basal gastrin secretion in the *in vitro* tissue test. It therefore seems very unlikely that the inhibitor acts via releasing somatostatin. Rather, it may act via a novel mechanism which is not part of the normal physiological control of gastrin release.

The inhibitor may act at the post-receptor level, for example, by inhibiting vesicle fusion. Its mechanism of action may be similar to that of clostridial neurotoxins, which inhibit neurotransmitter release by damaging the SNARE proteins involved in vesicle fusion (Williamson *et al.,* 1996; Gil *et al.,* 1998; Chaddock *et al.,* 2000). The inhibitory factor in rumen fluid and in the hypoacidic abomasum may have a similar mode of action on the G cell. Before the mode of action of the bacterial inhibitor can be determined it must first be isolated and characterised.

Future directions.

There are two outstanding issues from these studies which warrant further research. These are the effect of inflammation and tissue gastrin depletion on serum gastrin levels during abomasal parasitism, and the identity of the microbial product which inhibits gastrin secretion. Reduced amidated gastrin concentrations due to tissue gastrin depletion could be studied by examining the concentration of glycine extended gastrin intermediates in serum. The hypersecretion of gastrin results in a reduced residency time of gastrin in the G cell, which in turn increases the concentration of glycine extended gastrins in the blood and antral tissue. Thus, when depletion of gastrin occurs during gastrin hypersecretion, it would be expected that circulating glycine extended gastrin concentrations would be increased, even when amidated gastrins are decreased.

The next step in the study of the bacterial inhibitor of gastrin secretion is its separation and isolation. Although the hydrophobic nature of activity has made purification difficult, the biggest constraint in separation was the necessity to use a bioassay to assess separation fractions. This has restricted the use of detergents and organic solvents. Before further chemical analysis of the inhibitory compound can be conducted, it will have to be separated and suspended in a medium that allows the active fraction to be tested using the tissue assay.

CHAPTER 8

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APPENDIX 1

Amidated Gastrin Radioimmunoassay

This assay is a modification of the method described by Hansky and Cain (1969) described by Simpson *et al.* (1993).

Assay Buffer: 0.02M Veronal buffer, with 0.5% BSA, 0.04% thiomersal, 0.004% neomycin, pH8.6.

Per litre:

4.12g Na barbiturate (Reidel-de Haën, Germany)

0.744g barbitone (BDH Chemicals Ltd, Poole, England)

5g bovine serum albumin (Boehringer Mannheim, Fraktion V)

400mg thiomersal (ethylmercuri-thiosalicyclic acid, sodium salt Acros organics, NJ, USA)

40mg neomycin sulphate (N1876 Sigma co, St Louis, USA).

Tracer: Synthetic non-sulphated human G17 (Research Plus, Bayanne, NJ, USA) was labelled with ^{125}I using the chloramine T method. The label was isolated on a Sephadex G10 column and purified on DEAE cellulose column with a NaCl gradient from 0 to 1M. The tracer for the assay contained 1200-1800cpm per 500µL.

Antiserum: Ab74 (the kind gift of Dr. J Hansky) which has equal affinity for human, porcine and ovine G14, G17 and G34 was used at a final dilution of 1:100 000 with 1:4000 normal rabbit serum (NRS). 400µL antibody solution was added to the assay, making a final dilution of 1:40 000 Ab and 1:2500 NRS.

Standards: Synthetic non-sulphated human G17 was diluted in assay buffer to concentrations of 2.5, 5, 10, 20, 50, 100, 200, 400pM.

Second antibody: Serum from sheep immunised against rabbit gamma-globulin was used as a precipitating second antibody.

This was standardised against Donkey anti-rabbit globulin (IDS England).

Assay procedure: All samples and standards were assayed in triplicate. Assay tubes contained:

(i) Totals- 500µL tracer

(ii) NSB -100µL buffer, 400µL NRS w/o Ab, 500µL tracer.

(iii) Standards - 100µL standard 400µL Ab, 500µL tracer.

(iv) Samples - 100µL sample 400µL Ab, 500µL tracer.

The tubes were incubated for two days at 4°C. 200µL second antibody was added to all tubes except totals and incubated for a further three days at 4°C. Tubes were centrifuged at 2000*g* for 30 minutes, the supernatant discarded and the pellet radioactivity quantified for 5 minutes in a gamma counter.

APPENDIX 2

Somatostatin Radioimmunoassay

Assay buffer: Veronal buffer, 0.02M, 0.004M acetate, 0.5% BSA, 0.1% thiomersal, 1% trasylol, pH7.4. Per litre:

4.9g Na barbital (Reidel-de Haën, Germany)

0.32g Na acetate

5g BSA (Boeringer Mannheim, Fraktion V)

100mg Thiomersal (ethylmercuri-thiosalicyclic acid, sodium salt Acros organics, NJ, USA)

10mL Trasylol

Tracer: ^{1}Tyr-somatostatin (Peninsular Laboratories, Cal., USA) was labelled with ^{125}I using chloramine T method. On the day of the assay, the label was purified on CM-cellulose (CM 52) column with ammonium acetate buffer gradient from 0.002 to 0.2M NaCl. The tracer for assay contained c3500cpm per 100µL.

Antiserum: Monoclonal antibody Soma 03 (the kind gift of Prof. C.H. McIntosh) was used at a dilution of 1:4 000 000, with 400µL added to assay tubes, giving a final dilution of 1:1 000 000.

Standards: Somatostatin was dissolved in 0.1M acetic acid containing 0.05% BSA to give a final dilution of 2µg/50µL and stored lyophilised. For the assay, 100µL distilled H_2O followed by 400µL assay buffer were added to give a final concentration of 10µg/mL. This was then diluted in assay buffer to concentrations of 2.5, 5, 10, 20, 50, 100, 200, 400pM.

Assay procedure: All samples and standards were assayed in triplicate, in glass tubes. Assay tubes contained:

(i) Totals - 100µL tracer

(ii) NSB -300µL buffer, 100µL tracer.

(iii) Standards - 100µL each of standard, buffer, Ab and tracer.

(iv) Samples - 100µL each of sample, buffer, Ab and tracer.

Tubes were incubated at 4°C for three days. Dextran T70 was dissolved in 0.05M phosphate buffer pH7.5 then activated charcoal (Norit, 1,25g/100mL) and hormone free plasma (100µL/100mL) were added and solution mixed for one hour. Aliquots of 1mL were then added to all tubes except totals, mixed, allowed to stand for 15 minutes then centrifuged at 2000*g* for 30 minutes. The supernatant was then discarded and the radioactivity of the pellet quantified for 5 minutes in a gamma counter.

APPENDIX 3

Pepsinogen assay

Pepsinogen concentrations were estimated using a previously validated method (Scott *et al.*, 1995) modified for assay of small sample volumes.

Duplicate tubes containing 100μL of sample or standard and 150μL of substrate (3.2% BSA, fraction V, 1% glycine, pH1.6) were incubated for 30 minutes at 37°C before 500μL of 10% perchloric acid was added to one tube. The other tube was then incubated for a further three hours before the addition of the same amount of perchloric acid. Undigested substrate was pelleted by centrifugation at 10 000*g* for 10 minutes and 20μL of the supernatant added to 200μL of a mixture of bicinchoninic acid (BCA) and $CuSO_4$ (50 parts 10% BCA to 1 part 4% $CuSO_4$) in quadruplicate in flat-bottomed microtitre plates.

After incubation for 30 minutes at 37°C, the O.D. was read at 550 nanometers. Pepsinogen concentrations were estimated by comparison with standards containing L-tyrosine (0, 1.8 and 3.6 mM) and expressed in i.u.

Errata

Page 38 line 29: replace **Abbot** with **Abbott**.

Page 39 line 28: replace **gastrin** release with **pepsinogen** release

Page 53 line 3: replace **in vitro** with ***in vitro***.

Page 57 line 7: Pentobarb 500 is the tradename for Pentobarbitone 500mg.mL^{-1}

Page 57, Section 2.2.1.3: Insert gastrin assays were conducted as described in Appendix 1 and somatostatin assays as in Appendix 2.

Facing Page 59, Table 2.1: replace **hGRP** with **GRP**

Page 79 line 9: replace **3.2.2.1** with **3.2.1.1.**

Page 80 line 14: replace **3.2.2.2** with **3.2.1.2**

Page 82 line 27: replace **second** infection with **first** infection

Page 86 line 3: replace **though** with **through**

Page 87 lines 11-12 replace **by** correlation with **of** correlation

Facing Page 102, Fig 4.9, line 5: replace **rr** with r^2

Page 105 line 28: replace **LPT-2** with **LPI-2**

Page 112 line 14: replace **ABI-1** with **AI-1**

Page 114 line 16: replace **ABI-1** and 2 with **AI-1** and 2

Page 117 lines 15 and 17: replace RUM-14 to **RI-17** with RUM-14 to **RUM-17**

Page 117 lines 22, 23 and 24: **incubates** should read **rumen incubates**

Page 118 line 7: replace Section **4.2.4.1** with Section **4.3.2.5**

Page 118 line 14: replace **RUM-26** to -26 with **RUM-24** to -26

Page 151 line 6: replace **activity was present** with **breakdown activity was not present**

Page 151 line 7: **freeze** should read **freeze-dried**

Page 157 line 23: replace **of** the fundus with **or** the fundus

Page 159 line 27: should read elevation of serum **gastrin**

Page 160 line 16: should read "**and it is only present**," not "**the it is only present**"

Page 164 line 4: replace **Abbot** with **Abbott**

Page 191 line 5: replace ***Australian*** with ***Asian-Australian***

Page 200, add: Simpson, HV (2000). Pathophysiology of abomasal parasitism: is the host or the parasite responsible? *The Veterinary Journal*, **160**, 177-191.