ACTA UNIVERSITATIS AGRICULTURAE SUECIAE

VETERINARIA 69

Studies of Gastrin and Gastric Secretion in the Horse

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av

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Akademisk avhandling som för vinnande av veterinärmedicine doktorsexamen kommer att offentligen försvaras i Ettans föreläsningssal, Klinikcentrum, SLU, Uppsala, torsdagen den 27 januari, 2000, kl 9.15.

Fakultetsopponent:	Professor Al Merritt, University of Florida, USA
Disputations-ordförande:	Docent Göran Dalin, SLU, Uppsala
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The amino acid sequence of horse gastrin G 17 and G 34 differs from other species in several respects. In particular, horse gastrin contains a lysin instead of a glutamic acid in the acidic stretch of amino acids preceding the bioactive C-terminal portion of the molecule, which makes the molecule less acidic than in gastrins of other species. Determination of the cDNA indicates that horse preprogastrin contains three more amino acids than preprogastrins from other species. Horses do not express sulphated gastrins and probably lack the required enzyme system. In comparison with other species a relatively large amount of G 34 is found in the horse antrum. Horse gastrins do not bind well to antibodies produced against human gastrin.

Gastrin release in horses seems to be little influenced by nervous excitation. Following local stimulation of the gastric antrum by a meal, a significant rise of the plasma gastrin concentration is found. A grain meal produces a more prolonged gastrin release than a hay meal, possible due to the prolonged emptying of the grain meal from the stomach.

The synthesised horse gastrin 17 effectively stimulates the gastric acid output in horses, dogs and rats. Horses are extremely sensitive to histamine stimulation. Thus, dogs require 4-8 times more and rats 250-500 times more of histamine to produce maximal acid secretion.

Nervous stimulation by teasing evokes a minute acid response in horses. Insulin-induced hypoglycaemia in contrast to most other species reduces the basal acid output in the horse which is in contrast to the effect seen in other species. Basal horse gastric acid secretion is stimulated by cholinergic stimulation.

Keywords: Atropine, bethanechol, cortisol, equine, gastric acid secretion, gastric cannula, gastrin, histamine, insulin, teasing, volume secretion

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Uppsala, 1999
ISSN 1401-6257
ISBN 91-576-5908-7

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Doctoral thesis
Swedish University of Agricultural Sciences
Uppsala 1999

Acta Universitatis Agriculturae Sueciae
Veterinaria 69

ISSN 1401-6257
ISBN 91-576-5908-7

Tryck: SLU Service/Repro, Uppsala 1999

Om en människa kunde korsas med en katt
skulle det förbättra människan men försämra katten

Mark Twain

Abstract

Sandin, A. *Studies of gastrin and gastric secretion in the horse.*
Doctor's dissertation.
ISSN 1401-6257, ISBN 91-576-5908-7

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Contents

Appendix

Papers I-IV

The present thesis is based on the following papers, which will be referred to by their Roman numerals:

I. Johnsen A.H., Sandin A., Bundgaard J.R., Rourke I., Nilsson G. and Rehfeld J.F. 1998. Unique progastrin processing in equine G-cells suggets marginal tyrosyl transferase activity, *Eur J Biochem 255,* 432-438.

II. Sandin A., Girma K., Sjöholm B., Lindholm A. and Nilsson G. 1998. Effects of differently composed feeds and physical stress on plasma gastrin concentration in horses, *Acta vet Scand 39,* 265-272.

III. Sandin A., Andrews F.M., Nadeau J.A. and Nilsson G. Effects of horse gastrin on gastric acid secretion in horses, dogs and rats. *Submitted.*

IV. Sandin A., Andrews F.M., Nadeau J.A. and Nilsson G. 2000. Effect of nervous excitation on acid secretion in horses. *Acta Physiol Scand.* In press.

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Abbreviations

The following abbreviations are used in this thesis:

Ab	antibody
Ala	alanine
bwt	body-weight
bp	base pair
cAMP	cyclic adenosine monophosphate
CCK	cholecystokinin
CCK-PZ	cholecystokinin-pancreozymin
CGRP	calcitonin gene-related peptide
cDNA	complementary deoxyribonucleic acid
DNA	deoxyribonucleic acid
ECL cell	entero-chromaffin-like cell
FPLC	fluid-pressure liquid chromatography
G 17	gastrin 17
G 34	gastrin 34
Glu	glutamic acid
GRP	gastrin releasing peptide
HoG17	horse gastrin 17
HPLC	high-pressure liquid chromatography
HuG17	human gastrin 17
Lys	lysine
Met	methionine
PACAP	pituitary adenylate-cyclase activating peptide
PCR	polymerase chain reaction
RNA	ribonucleic acid
TAO	total acid output
Tyr	tyrosine
VIP	vasoactive intestinal polypeptide

Introduction

Background

Ulcer disease in the upper gastrointestinal tract of man is well documented. However, gastric ulcers have also been found in rats (Singer, 1913), guinea pigs and cats (Bolton, 1915-1916), rabbits (Rosenow, 1923), foxes (Shillinger, 1929), dogs (Volini, Widenhorn & Defoe, 1938), sheep (Hutyra, Marek & Manninger, 1941), cattle (Bartlett & Fincher, 1956), monkeys (Brady, 1958), mink (Gorham & Farell, 1958), pigs (Kowalczyk *et al.*, 1960), pinnipeds (Sweeney, 1974), ferrets (Andrews, Illman & Wynne, 1976), dolphins (Greenwood, Taylor & Wild, 1978), killer whales (Ridgway, 1979), llamas (Fowler, 1989; Johnson, 1989), elephants (Van Aswegen *et al.*, 1994), and horses (Rooney, 1964; Hammond, Mason & Watkins, 1986). Studies performed on a Swedish necropsy material (Sandin *et al.*, 1999; 2000) show that gastric ulcer disease in several breeds of horses has been common in Sweden during the last 75 years of this century.

The principal factor causing gastric ulceration seems to be the presence of acid in the stomach, although mucosal infections with microorganisms, such as *Helicobacter pylori* (Warren & Marshall, 1983), may also contribute to the development of ulcers. Different forms of Helicobacter bacteria have been isolated from the gastrointestinal tract of man, a non-human primate, cheetah, dog, cat, ferret, mouse, pig, bird, rat and hamster (Skirrow, 1994; Wesley, 1996). However, no reports of Helicobacter-related gastric ulcer disease in the horse have so far appeared in the literature.

Our knowledge about mechanisms regulating gastric acid secretion mainly originates from studies on dogs, cats, rats and man, but also from investigations on isolated tissues from the gastric mucosa of rabbits and other species. Such studies have revealed several differences between species. Only a few studies have been performed on the mechanisms regulating acid secretion in horses (Campbell-Thompson, 1994; Campbell-Thompson & Merritt, 1990; Kitchen, 1997; Kitchen, Merritt & Burrow, 1998).

Discovery and purification of gastrin

Edkins (1905; 1906) originally suggested that pig gastric antrum contained a factor that stimulated acid secretion and he named it gastrin. The gastrin hypothesis was later doubted by several researchers (Dale & Laidlaw, 1910; Zeljony & Savich, 1911; Popielski, 1912) and the stimulatory effect of the antral extracts was instead ascribed to histamine (Sacks *et al.*, 1932). However, at the end of the 1930s, Komarov (1938) demonstrated that histamine-free extracts prepared from the antral mucosa still effectively stimulated acid secretion. He also showed that their stimulatory effects were abolished when the extracts were treated with a protein-digesting enzyme, indicating that the stimulatory factor was protein in nature.

In 1964, Gregory & Tracy finally purified and sequenced gastrin obtained from pigs. It was found to be a heptadecapeptide (G 17) and to be present in the antral mucosa in two forms, with (G 17 I) and without (G 17 II) sulphate on the tyrosine residue.

Using antibodies directed towards gastrin, Yalow & Berson (1970) later detected an immunoreactive material of larger molecular size than G 17 in the antrum. It was found to be a molecular extension of the heptadecapeptide, having 34 amino acids (G 34) and it was also present in two forms - with and without tyrosine sulphation (Gregory & Tracy, 1972; Yalow & Berson, 1972).

Further gastrin radioimmunoassay work revealed the existence of gastrins that were smaller than G 17. Thus, G 14, also called minigastrin, was found in a human gastrinoma (Gregory *et al.*, 1979), G 6 in pig antrum (Gregory *et al.*, 1983), G 5 in muscle and brain from dogs (Shively *et al.*, 1987) and G 4, called tetrin, in gastrointestinal tissues from monkeys and pigs (Rehfeld & Larsson, 1979; Larsson & Rehfeld, 1979). Yalow & Wu (1973) found gastrin-like immunoreactivity in plasma from humans, pigs and dogs which indicated the existence of a gastrin peptide larger than G 34. It was named "big big gastrin". Rehfeld & co-workers (1974) also found such immunoreactivity in human serum and named it Component I.

Most of the different gastrin forms present in the antrum have also been demonstrated in plasma. In addition, an N-terminal form of gastrin, lacking the last 4 C-terminal amino acids, has been demonstrated in pigs (Gregory *et al.*, 1964; Dockray & Walsh, 1975).

Following the final purification of gastrin from pigs, the hormone has been extracted from several species and structural variations have been documented. Table I illustrates the structures of heptadecapeptides isolated from 13 species.

Inter-species differences have also been found regarding the degree of sulphation. In dogs, 24 % of the antral gastrin is sulphated (Andersen, 1985), in humans 50 % (Lamers *et al.*, 1982) and in kangaroos as much as 95 % (Johnsen & Schulkes, 1993).

The human gastrin gene is 4100 bp long and it is located on chromosome 17. It contains two introns, one proximal to the initiator codon and one in the region coding for G 34 (Ito *et al.*, 1984).

All the gastrin peptides having an intact C-terminal portion stimulate acid secretion. G 17 and G 34 have the same stimulatory potency (Eysselein *et al.*, 1984), although the secretion caused by G 34 is of somewhat longer duration. The longer duration may be explained by the somewhat longer plasma half-life of the G 34 peptide. In humans, the half-lives of G 17 and G 34 have been found to be 6 and 24-36 min, respectively (Eysselein *et al.*, 1984). The corresponding values for dogs are 3 and 9-15 min (Straus & Yalow, 1974). The presence or absence of sulphate on the tyrosine residue does not seem to influence the ability of the gastrin molecule to stimulate acid secretion (Gregory, 1979).

Table I. Amino acid sequences of gastrin from thirteen species

ANIMAL	AMINO ACID SEQUENCE	REFERENCE
Horse	Gln-Gly-Pro-Trp-Leu-Glu-Lys-Glu-Glu-Ala-Ala-Tyr-Gly-Trp-Met-Asp-Phe	Johnsen *et al.* 1998
Man	Gln-Gly-Pro-Trp-Leu-Glu-Glu-Glu-Glu-Glu-Glu-Ala-Tyr-Gly-Trp-Met-Asp-Phe	Bentley *et al.* 1966
Monkey	Gln-Gly-Pro-Trp-Met-Glu-Glu-Glu-Glu-Ala-Ala-Tyr-Gly-Trp-Met-Asp-Phe	Yu *et al.* 1991
Pig	Gln-Gly-Pro-Trp-Met-Glu-Glu-Glu-Glu-Glu-Ala-Tyr-Gly-Trp-Met-Asp-Phe	Yoo *et al.* 1982
Cow	Gln-Gly-Pro-Trp-Val-Glu-Glu-Glu-Glu-Ala-Ala-Tyr-Gly-Trp-Met-Asp-Phe	Lund *et al.* 1989
Sheep	Gln-Gly-Pro-Trp-Val-Glu-Glu-Glu-Glu-Ala-Ala-Tyr-Gly-Trp-Met-Asp-Phe	Kenner & Sheppard 1973
Goat	Gln-Gly-Pro-Trp-Val-Glu-Glu-Glu-Glu-Ala-Ala-Tyr-Gly-Trp-Met-Asp-Phe	Bonato *et al.* 1986*a*
Dog	Gln-Gly-Pro-Trp-Met-Glu-Glu-Glu-Glu-Ala-Ala-Tyr-Gly-Trp-Met-Asp-Phe	Gantz *et al.* 1990
Cat	Gln-Gly-Pro-Trp-Leu-Glu-Glu-Glu-Glu-Ala-Ala-Tyr-Gly-Trp-Met-Asp-Phe	Kenner & Sheppard 1973
Rat	Gln-Arg-Pro-Pro-Met-Glu-Glu-Glu-Glu-Glu-Ala-Tyr-Gly-Trp-Met-Asp-Phe	Fuller *et al.* 1987
Rabbit	Gln-Gly-Pro-Trp-Leu-Gln-Glu-Glu-Glu-Glu-Ala-Tyr-Gly-Trp-Met-Asp-Phe	Jiang *et al.* 1988
Guinea pig	Gln-Gly-Pro-Trp-Ala-***-Glu-Glu-Glu-Ala-Ala-Tyr-Gly-Trp-Met-Asp-Phe	Bonato *et al.* 1986*b*
Chinchilla	Gln-Gly-Pro-Trp-Ala-***-Glu-Glu-Glu-Ala-Ala-Tyr-Gly-Trp-Met-Asp-Phe	Shinomura *et al.* 1987

Underlinings indicate differences from horse gastrin. The references of pig, cow, dog and rat have been obtained from their cDNA and/or gene structure, whereas the rest have been identified by their amino acid sequences. *** indicate that gastrin from these species only have 16 amino acids.

Tissue distribution of gastrin

The highest concentrations of gastrin are found in the antral mucosa (McGuigan, 1968; Greider, Steinberg & McGuigan, 1972; Nilsson, Yalow & Berson, 1973). In the antrum of most species, the G 17 forms dominate over the G 34 forms. In the intestine, the gastrin concentrations gradually decrease (Nilsson, Yalow & Berson, 1973). The highest concentrations of extra-antral gastrins are found in the proximal portion of the duodenum. The distribution of the different molecular forms of gastrin in the duodenum differs somewhat from that in the antrum, showing a higher percentage of G 34 than of G 17 (Berson & Yalow, 1971).

Certain other extra-gastric sources of gastrin have been demonstrated, including the major part of the remaining portion of the gastrointestinal tract (Nilsson, 1980*b*), the pancreas (Blair *et al.*, 1969; Nilsson, Yalow & Berson, 1973; Bardram, Hilsted & Rehfeld, 1990), the central nervous system (Rehfeld, 1978*b*; Rehfeld *et al.*, 1984), the heart (Gersl, Goiny & Uvnäs-Wallensten, 1981) and the peripheral nerves (Uvnäs-Wallensten *et al.*, 1977; Lundberg *et al.*, 1978). It has also been shown that gastrin mRNA can be expressed in human spermatozoa (Schalling *et al.*, 1990).

Factors affecting the release of gastrin

Gastrin is released into the blood stream following nervous stimulation induced by various means. Thus, vagal excitation following sham feeding (Nilsson *et al.*, 1972), teasing (Feldman & Richardson, 1986), electrical stimulation of the vagal nerves (Uvnäs, 1942; Uvnäs, Uvnäs-Wallensten & Nilsson, 1975) and insulin-induced hypoglycaemia (Jaffe, 1970) releases gastrin. The vagal release is considered to be mediated by neurons releasing acetylcholine (Csendes, Walsh & Grossman, 1972) and GRP (McDonald *et al.*, 1978; Ekblad *et al.*, 1985).

When food has entered the stomach, distention of its antral portion (Pevsner & Grossman, 1955; Debas *et al.*, 1974) and chemical stimulation of the gastrin cells (Elwin, 1974; Lichtenberger, 1982; Strunz, Walsh & Grossman, 1978) further stimulate to the release of gastrin. How this release is mediated has not yet been clarified, but is facilitated by an antral pH higher than 3.

Certain mechanisms inhibit or suppress gastrin release. For example, when the antral pH is decreased (Woodward *et al.*, 1954; Nilsson *et al.*, 1972), gastrin release is reduced. Reduction of the antral pH will also cause the release of somatostatin from the antral D-cells which inhibits the gastrin cells in a paracrine way (Larsson *et al.*, 1979). There are also indications that the vagal nerves exert an inhibitory effect on the release of gastrin (Feldman *et al.*, 1979).

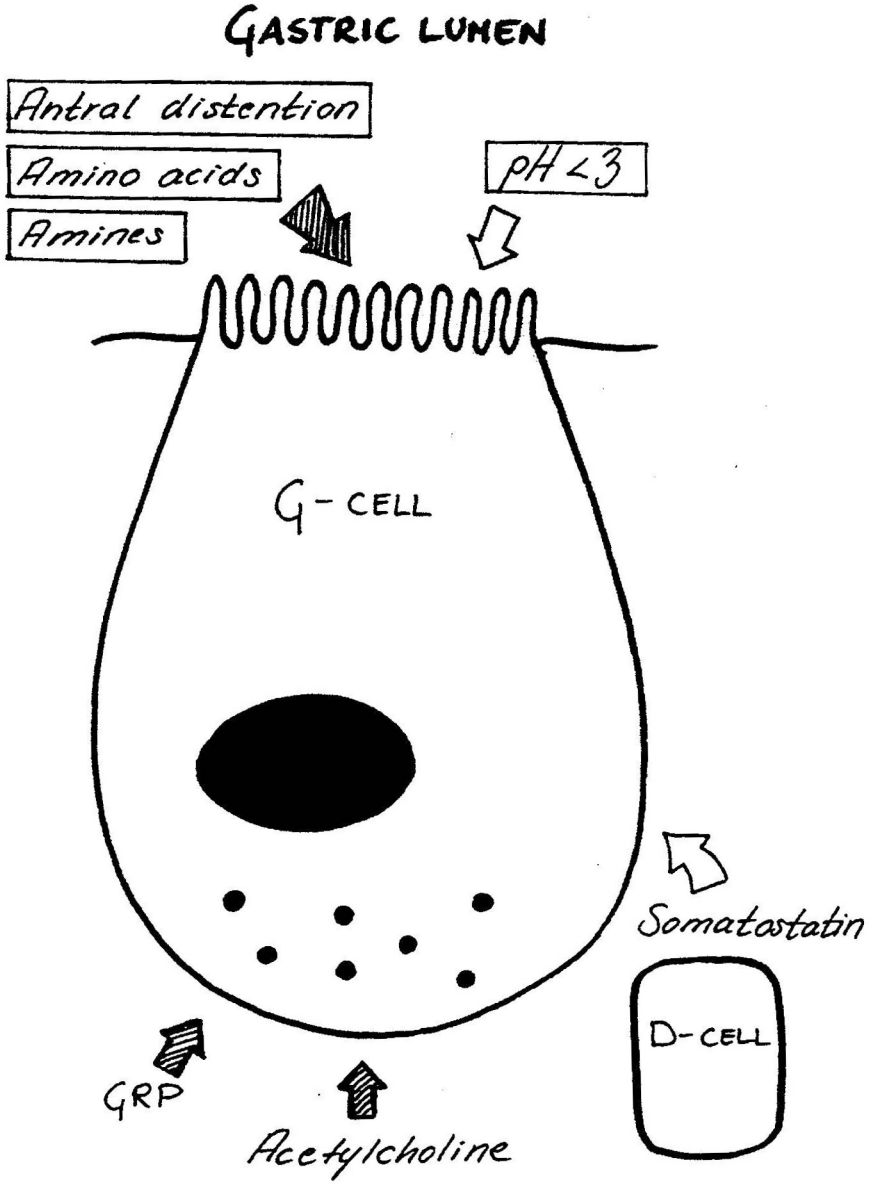

Figure 1. Factors causing stimulation (hatched arrows) and inhibition (open arrows) of gastrin secretion.

Effects exerted by gastrin and gastrin receptors

It is well established that gastrin stimulates gastric acid secretion. The released gastrin will bind to receptors on the ECL and parietal cells in the acid-secreting mucosa. In the ECL cells, the enzyme histidine decarboxylase will be activated by gastrin, which leads to the production of histamine (Håkanson *et al.*, 1974). Gastrin also stimulates the release of histamine (Thunberg, 1967; Ehinger *et al.*, 1968; Håkanson *et al.*, 1976; Håkanson *et al.*, 1986) together with cholinergic vagal excitation (Bergqvist *et al.*, 1980) and PACAP (Lindström *et al.*, 1997). The stimulatory effect of gastrin on the parietal cells, as demonstrated in *in vitro* experiments, is relatively weak compared to the effect of histamine (Berglindh, Helander & Öbrink, 1976; Soll, 1978).

Gastrin also exerts a trophic effect on the acid secreting mucosa (Johnson, Aures & Yuen, 1969). This trophic effect of gastrin probably facilitates healing of gastric ulcers.

Larger amounts of gastrin inhibit the acid secretion (Gillespie & Grossman, 1963) and such inhibition seems to be mediated by somatostatin (Bengtsson, Lundqvist & Nilsson, 1989) from D-cells located close to the parietal cells (Larsson *et al.*, 1979). No specific gastrin receptor mediating such inhibition has yet been identified. Instead, there are two variants of CCK-receptors. The CCK-A receptor, cloned from rat pancreas, has an approximately 1000-fold higher affinity for CCK than for gastrin (Wank *et al.*, 1992). The CCK-B receptor, or the gastrin receptor, on the other hand, seems to have the same affinity for both peptides, *Kd* being 3.6×10^{-10} (Soll *et al.*, 1984). The amino acid sequence of the gastrin receptor has been deduced after expression cloning of a canine parietal cell cDNA library by Kopin *et al.* (1992) and it has the typical features of a G-protein-binding 7-transmembrane receptor. Activation of this receptor produces an increase in intracellular Ca^{2+} and inositol triphosphate concentrations.

Mechanisms stimulating acid secretion

Gastric acid secretion is regulated by a number of factors that act in a stimulatory or inhibitory manner. Under normal conditions, these factors balance each other.

The parietal cells are believed to be activated by direct cholinergic, histamine and gastrin stimulation. Gastrin will reach the parietal cells via the vascular system, whereas histamine stimulates them in a paracrine way.

Several animals, including horses (Campbell-Thompson & Merritt, 1986) rats, ferrets (Pfeiffer & Peters, 1969) and hens (Friedman, 1939) have a continuous basal secretion of acid. In other species, for example man and dogs, basal secretion may be absent or is intermittent of varying amount. To our knowledge, there are no reported studies elucidating the specific mechanisms responsible for this continuous basal gastric acid secretion. However, it has

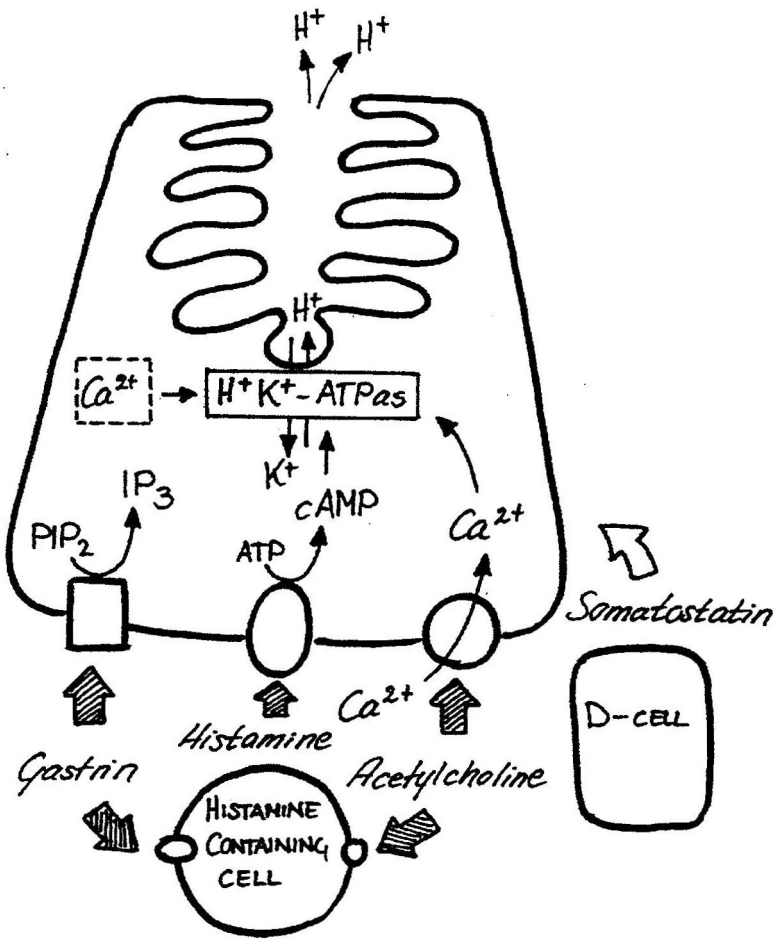

Figure 2. Mechanisms stimulating (hatched arrows) and inhibiting (open arrow) the secretion of hydrogen ions from the parietal cells.

been shown that basal gastric acid secretion is inhibited by anticholinergic drugs in rats (Pendleton, Miller & Ridley, 1980) and hens (Friedman, 1939; Long, 1967). Furthermore, H_2-receptor antagonists, such as ranitidine and famotidine, inhibit basal acid secretion in rats (Seensalu *et al.*, 1990; Okabe *et al.*, 1990) and horses (Campbell-Thompson & Merritt, 1987; Murray & Grodinsky, 1992). It has also been demonstrated that the basal acid secretion in rats may be inhibited by a novel CCK-B/gastrin receptor antagonist (Amagase, Ikeda & Okabe, 1999). Thus it seems that the mechanisms considered to activate gastric secretion during a meal also cause the continuous gastric acid secretion between the meals.

The stimulation of acid secretion is traditionally divided into three phases. During the cephalic phase, vagal excitation induced by the smell, sight or taste of the food starts the secretion. When the food reaches the stomach, the gastric phase starts and the acid output will increase further, due to the elevated gastrin release and the mechanical stimulation of the acid-secreting portion of the stomach. It has been claimed that some stimulation of acid secretion also originates from the intestine (Gregory & Ivy, 1941; Sircus, 1953). The magnitude of this intestinal phase is difficult to determine, but it has been estimated to be 5-10 %. The mechanism behind this intestinal stimulation is not well understood.

Intracellular activation of the parietal cell

Histologically, the resting parietal cell differs in appearance from the activated cell. Following stimulation, the canaliculi of the resting cell are converted into secretory units with tubulovesicles located in the wall of the canaliculi. These tubulovesicles contain the H^+/K^+-ATPase. Following activation of the H_2-receptors on the parietal cell surface, the intracellular cyclic AMP system is stimulated. When gastrin stimulates the cell, an intracellular release of inositol triphosphate and Ca^{2+} takes place. The cholinergic activation of the cell alters the permeability of the cell membrane to ionised calcium and the calmodulin regulated Ca^{2+} pump extrudes Ca^{2+} from the cell. The final event in the stimulation process includes an activation of the H^+/K^+-ATPase enzyme, which results in the secretion of hydrogen ions into the secretory canaliculi. The related events are illustrated in figure 2.

Mechanisms inhibiting gastric acid secretion

During a meal, when the gastric content becomes more acidic or if acid secretion occurs between meals, a number of inhibitory mechanisms will be activated. Lowering of the intra-antral pH reduces the secretion of gastrin, which will decrease the stimulation of the ECL and parietal cells. When the gastric acid content enters the first portion of the duodenum, another pH-sensitive inhibitory mechanism is activated, which seems to interfere with the acid secretion at the parietal cell level by releasing a putative hormone, bulbogastrone

(Nilsson, 1980*a*). In addition, fatty acids in contact with the duodenal mucosa evoke acid inhibition (Kosaka & Lim, 1930), possibly mediated by CCK-PZ released from the duodenal and jejunal mucosa (Gillespie & Grossman, 1964). Whether the related inhibitory mechanisms operate in horses has not been demonstrated so far.

Aims of the thesis

The high frequency of gastric ulcer disease in horses and the lack of information about the mechanisms that regulate gastric acid secretion in this species form the basis for this study.

The research work was carried out in order to

1) determine the cDNA for horse gastrin

2) isolate and determine the amino acid structure of two horse gastrins, G 17 and G 34

3) determine the degree of sulphation of gastrin

4) study the quantitative distribution of different molecular forms of gastrin in the antrum

5) investigate the release of gastrin during fasting, under different feeding conditions and during exercise on a treadmill

6) synthesise the horse G 17 and

7) determine its biological activity on gastric acid and volume secretion in the horse and compare these effects with those of pentagastrin and histamine

8) compare the same effects of HoG17 with those of human gastrin, pentagastrin and histamine in the dog and rat and

9) study the effect of nervous excitation and the cholinergic influence on the gastric acid output and the volume secretion in horses.

Materials and Methods

Analytical and preparative studies on horse gastric tissue (Paper I)

Antral mucosae from healthy Standardbreds *(Equus caballus)* were obtained from a local slaughterhouse. Following dissection, the mucosae were frozen on solid CO_2 and stored at -80°C.

Analytical investigations of antral tissues

Extracted pieces of frozen antrum were sectioned into small pieces and then placed in boiling water (10 ml water/g tissue) for 20 min. The pieces were then homogenised using an Ultra-turrax blender, and centrifuged for 10 min at 10000 *g*. The precipitate was re-extracted with ice-cold 0.5 M acetic acid (10 ml/g tissue). The supernatants obtained from the two extractions were analysed separately by gel chromatography, using 1 cm x 100 cm columns packed with Sephadex G-50 Superfine (Pharmacia). The samples were eluted in 1.1 ml fractions at a rate of 4.0 ml/h at 4°C with 20 mM barbital (pH 8.4), containing 0.1 % bovine serum albumin. Calibration was performed using ^{125}I-albumin (V_O) and ^{22}NaCl (V_t). The elution constants (K_d) of peaks eluting at V_e were calculated as $K_d=(V_e-V_O)/V_t-Vo)$.

Anion-exchange chromatography was performed on an FPLC system (Pharmacia) using a 5 mm x 50 mm MonoQ column (Pharmacia). The column was eluted (1 ml/min) at 20°C with a gradient ranging from 0-450 mM NaCl for 60 min in a buffer containing 50 mM Tris/HCl, pH 8.2, and 10 % acetonitrile. One ml fractions were collected.

Preparative investigations of antral extracts

In all, 80 g from three different antra were used. The tissue was ground in a mortar at -20°C and the powder obtained was then extracted twice in boiling water (5 ml/g tissue), after which the three supernatants were pooled.

Before sequencing of the horse gastrins, the pooled supernatants were subjected to a series of purification steps carried out on various columns. First, the frozen water extracts were thawed and centrifuged and then loaded onto a preparative SepPak C8 cartridge (10 g in a 35 ml syringe) at 10 ml/min. The immunoreactivity was contained in the last eluate and the volume was then reduced in a rotavapor and the concentrate was diluted with an equal volume of 0.1 % trifluoroacetic acid before pumping onto a 4.6 mm x 250 mm C4 reverse-phase column (Vydac). Following equilibration with a 10 % solvent of 0.1 % trifluoroacetic acid in acetonitrile, the column was eluted with a gradient from 10 % to 50 % of this solvent for 40 min.

Before the next purification step, the pH of the fractions containing gastrin immunoreactivity was adjusted to 8.0 and subjected to anion-exchange chromatography, using a MonoQ column on an FPLC-system. The peptides were further purified in three reverse phase HPLC steps. Details are given in Paper I.

Determination of gastrin in antral extracts

Gastrin in antral extracts was determined by a radioimmunological method described elsewhere (Rehfeld, Stadil & Rubin, 1972). Antibodies directed towards different parts of the gastrin molecule and with different abilities to detect sulphated and non-sulphated gastrins were used.

Antibodies 2604, 2605 and 2609 were made against synthetic non-sulphated HuG17 (Rehfeld, Stadil & Rubin, 1972). The Ab 2609 is specific for the carboxyamidated C-terminal tetrapeptide which is common for both gastrin and CCK. This Ab does not discriminate well between the two hormones (Rehfeld, 1978*a*). Antibodies 2604 and 2605 are specific for gastrin, having a very low (<1 %) cross-reactivity with CCK. Ab 2604 binds sulphated and non-sulphated gastrins equally well, whereas Ab 2605 has only a low (5 %) cross-reactivity with sulphated gastrin. Ab 92128 is specific for sulphated CCK (Rehfeld, 1998) and Ab 8017 is directed against the N-terminus of HuG17 (Bardram & Rehfeld, 1988). Gly-extended gastrin was measured with Ab 7270 and the total of Gly-extended and precursors extended further at the C-terminus was measured with the same antiserum after cleavage with trypsin and carboxypeptidase B (Hilsted & Rehfeld, 1986).

Mass spectrometry and sequence analysis

After final purification, the peptides were analysed in a matrix-assisted laser-desorption/ionization time-of-flight mass spectrometer (Biflex) in the linear mode. All samples were analysed in both the negative and positive modes and the spectra were recorded for 20-100 laser shots.

Before sequencing, pyroglutamate aminopeptidase was added to the sample to split the pyroglutamate residue. The amino acid sequences were then determined using an automatic protein sequencer (model 494A, Perkin Elmer ABD) equipped with an on-line HPLC-system for detection of the amino acid phenylthiohydantoin derivatives.

Rapid amplification of cDNA ends (RACE) PCR

For methodological details, see Paper I. Oligonucleotides for PCR were produced using an Applied Biosystems 392 DNA/RNA synthesiser. Messenger RNA was isolated from horse antral mucosae using the QuickPrep Micro procedure (Pharmacia).

cDNA cloning and sequencing

The RACE PCR products were subjected to electrophoresis on a 1 % agarose gel. The DNA bands were liberated from the gel using the Sephaglas BandPrep procedure (Pharmacia) and cloned directly into the pMOS*Blue* T-vector (Amersham). Thereafter, the recombinant horse cDNA fragments were sequenced using the ABI Prism Dye Terminator Cycle Sequencing Ready reaction system (Perkin Elmer) and the reactions resolved on an ABI Prism 377

DNA sequencer (Perkin Elmer). Both strands of the PCR clones were sequenced to confirm the sequence data.

Expression of equine gastrin in HIT cells

Full-length horse gastrin cDNA was amplified by PCR from cDNA and the PCR product was digested with *Hind* III and *Eco*RI. The cDNA was ligated into the corresponding sites of the pcDNA3.1/Zeo(+) vector (Invitrogen) to generate an expression vector. The cloned cDNA was sequenced and the plasmid DNA was purified before culture and transfection on HIT cells according to a method described by Bundgaard *et al.* (1996). Two days after transfection, the cells were harvested and the peptides were extracted in boiling water for 30 min. The samples were thereafter analysed by anion-exchange chromatography and by radioimmunoassays as described above. There was no background contamination, since untransfected HIT cells do not express detectable gastrin levels.

Plasma gastrin concentrations in relation to feeding and exercise in horses (Paper II)

Animals

Six clinically healthy Standardbreds (weight 447-526 kg) were used. Two weeks before the study, the horses were taken in from pasture and put on a diet consisting of hay and oats.

Feeding experiments

In these experiments, three differently composed meals were given. At one meal horses were allowed to eat an unlimited amount of hay, approximately 8-9 kg during 12.5 hours. The second meal consisted of a restricted amount of hay (0.6 kg /100 kg bwt), together with oats (0.2 kg/100 kg bwt) fed twice with a six hour interval. The third meal consisted of oats (0.2 kg/100kg bwt) only and it was also given twice with a 6 h interval. Water and trace mineralised salt were provided free choice for the experimental period. The animals were allowed a one-week recovery period between the experiments.

Treadmill experiments

In order to determine the effect of exercise on plasma gastrin concentrations, horses were subjected to running tests on a horizontal treadmill (Sikob, Sollentuna, Sweden). In one series of experiments, an incremental speed test was performed. After 2 min of walking on the treadmill, horses started to trot at a speed of 5 m/s. The velocity was increased by 1 m/s every 2 min up to 9 m/s. In another series of experiments, the horses ran at a constant speed (4-6 m/s) for 30 min. The speed was set to maintain a heart rate of 160-180 beats per min, using a Minogram 410 System (Siemens Elema, Sweden).

Determinations of gastrin and cortisol concentrations in plasma

Blood samples for determination of gastrin, cortisol and glucose were collected in heparinised tubes. Samples intended for gastrin and cortisol determinations were kept on ice before centrifugation. The collected plasma was then frozen and stored at -20°C until assayed. Whole blood glucose concentrations were determined immediately after collection of the blood.

Plasma gastrin concentrations were analysed using a radioimmunoassay method previously described by Nilsson (1975). Ab 4562, generously donated by Professor Jens Rehfeld, was used. This antibody recognises G 17 and G 34 to a similar extent, as well as sulphated and non-sulphated gastrins (Rehfeld, 1981). There is only a small 0.9-2.6 % cross-reactivity to CCK peptides. When postprandial plasma gastrin concentrations were superimposed on the standard curve, only small variations in parallelism were obtained. Plasma samples were assayed in duplicate.

Plasma concentrations of cortisol were measured with a commercial radioimmunoassay kit (TKCO5, Diagnostic Products Corp., Los Angeles, CA, USA). This kit has previously been validated for use in horses (Young & Smyth, 1988) and has been shown to have a low cross-reactivity with other steroid hormones present in the blood.

Acid secretion studies (Papers III and IV)

Animals (Papers III and IV)

Gastric acid secretion tests were carried out in conscious horses, dogs and rats. Horse experiments were carried out on five female mixed-breed horses, eight years of age and weighing 344-470 kg. At 4-6 months of age, they were provided with a gastric cannula according to a method described by Campbell-Thompson & Merritt (1986).

In dog experiments, four female and two male adult mongrel dogs weighing 24-29 kg and provided with a gastric cannula were used. The animals were kindly placed at our disposal by Astra Hässle AB in Gothenburg, Sweden where the experiments were performed.

In experiments on rats, six male Sprague-Dawley (B&K Universal AB, Sollentuna, Sweden) rats weighing approximately 600 g and fitted with a gastric cannula were used.

All animals were well adapted to handling before the start of the experiments and trained to stand with gentle restraints during the acid secretion experiments. All experimental studies in this thesis were approved by the University of Tennessee Animal Care and Concerns Committee (horses) and the Swedish Ethical Committees in Uppsala (horses and rats) and Gothenburg (dogs).

Acid secretion determinations (Papers III and IV)

Before the start of the gastric secretion experiments, animals were fasted for 18-22 hours, during which time they were allowed to drink water. Fourty-five minutes before the experiments were initiated, the gastric cannula stylet was removed to drain residual gastric contents. Basal gastric fluid was collected every 15 min for 45-60 min prior to the start of the stimulation tests. Gastric juice was collected every 15 min from horses and dogs and every 60 min from rats. After each collection, the gastric juice volume and pH were determined. The gastric juice acidity was measured by electrometric endpoint (pH 7.0) titration with 0.1 N NaOH in dogs and horses and 0.01 N NaOH in rats, using an automatic titrator (ABU 80, Radiometer, Copenhagen, Denmark). Total acid output was calculated for each sample by multiplying the gastric juice volume by the acidity. All measurements of acid parameters were performed in duplicate or triplicate.

In acid secretion experiments in horses using bethanechol as a stimulant, a copious amount of saliva was obtained that mixed with the collected gastric juice. Therefore, saliva was collected from the mouth. Its pH and buffering capacity were determined using a micro electrode (pHG200, Radiometer, Copenhagen, Denmark) and by estimating the amount of 0.01 N HCl required to lower the saliva pH to 3.1.

Determinations of bile acids in gastric juice (Paper III)

In order to obtain information about the magnitude of liquid reflux from the duodenum during acid secretion experiments, the amount of 3α-hydroxy bile acids was determined in the collected gastric juice, using an Enzabile®test kit (Nycomed, Oslo, Norway).

Determinations of glucose concentrations in whole blood (Paper IV)

Blood glucose concentrations were measured before and after insulin administration, using a digital glucose meter (Precision QID, Medisense Inc., Bedford, MA 01730, USA).

Synthesis of horse gastrin (Paper III)

The sequence of HoG17, Pyr-GPWLEKEEAAYGWMDF-NH_2, was synthesised using a solid phase peptide synthesiser. The synthesised molecule had a molecular weight of 2039.3 Da. The accuracy of the mass spectrometric analysis was 0.1 %.

Substances used to influence acid secretion (Papers III and IV)

Insulin (Humulin®Regular, Eli Lilly, Sweden) was given to induce hypoglycaemia, which is known to induce central-mediated vagal excitation (Roholm, 1930). Bethanechol chloride (5.15 mg/ml; Urecholine, Merck, West Point, PA, USA) is a cholinergic agonist that has been shown to stimulate acid secretion in most species. Atropine sulphate (15 mg/ml; Amvet Scientific Products,

Yaphank, NY 11980), an antagonist of muscarinic receptors, has been demonstrated to inhibit gastric acid secretion in several species.

The pentapeptide of gastrin (Peptavlon®, Zeneca, Sweden), HuG17 (Bachem, Bubendorf, Switzerland), synthesised HoG17 and histamine dihydrochloride (Sigma, Sweden), were also used to stimulate gastric acid secretion. In horses, the H_1-receptor antagonist pyrilamine maleate (Histavet-P, Schering-Plough, NJ, USA) was administered to prevent the circulatory and respiratory side effects seen during histamine administration.

Gastrin and histamine stimulation of acid secretion (Paper III)

Doses of HoG17, pentagastrin and histamine ranging from liminal to maximal were infused into all animals. In addition, HuG17 was given in dogs and rats. The horses received 1-16 μg/kg bwt/h of pentagastrin, 0.15-2.4 μg/kg bwt/h of HoG17 or 4-32 μg/kg bwt/h of histamine. The dogs were given 1-32 μg/kg bwt/h of pentagastrin, 0.15-4.8 μg/kg bwt/h of HoG17 or HuG17 or 8-256 μg/kg bwt/h of histamine. Corresponding doses in rats were 2-32 μg/kg bwt/h of pentagastrin, 0.3-4.8 μg/kg bwt/h of HoG17 or HuG17 or 250-4000 μg/kg bwt/h of histamine.

In dogs and horses, each stimulant was infused intravenously for 45 min and the dose was then doubled. In rats, each stimulant was infused subcutaneously for 60 min before doubling the dose. In horses, the H_1-receptor antagonist (0.1 mg/kg bwt/h) was infused continuously throughout the histamine experiment.

Vagal effects on acid secretion in horses (Paper IV)

Different modes of stimulation were used in order to determine the effect of nervous stimulation on gastric acid secretion. In one series of experiments, insulin hypoglycaemia was induced by the intravenous administration of insulin (0.15 IU/kg bwt). Before and during these 3.5 hour experiments, blood samples were collected every 15 min for determination of whole blood glucose concentrations.

In other experiments, the effects of the cholinergic agonist bethanechol (0.025 mg/kg bwt) on acid secretion were investigated. In still other investigations, the cholinergic antagonist atropine (0.06 mg/kg bwt) was given to determine its effect on the continuous basal acid secretion in the horse.

Horses were also stimulated by teasing, which means that the animals were allowed to smell and see, but not to taste, a grain mixture during a 15 min exposure period.

Results and Discussion

The high prevalence of gastric ulcers in horses and the limited knowledge about the physiological processes controlling gastric acid and gastrin secretions, constitute the main purpose of this study.

Following the pioneering work of Gregory & Tracy (1964) in sequencing the two porcine peptides G 17 and G 34, gastrin was isolated and its structure was determined in a large number of species (Table 1). The inter-species differences in structure have been reviewed in more detail by Johnsen & Rehfeld (1993).

Structural studies on horse gastrin (Paper I)

In the present work, two equine gastrin molecules were isolated and their structures were determined. A high degree of similarity with other mammalian gastrins was found. However, instead of a highly acidic stretch of amino acids preceding the bioactive carboxyamidated C-terminal heptapeptide, equine gastrin contains a remarkable substitution of Lys for Glu. In other mammalian species, this region exhibits a stretch of acidic residues, varying from three amino acids in the kangaroo (Johnsen & Shulkes, 1993), guinea pig (Bonato *et al.*, 1986*b*) and chinchilla (Shinomura, Eng & Yalow, 1987) to five amino acids in man (Bentley, Kenner & Sheppard, 1966). Horse gastrins are thus less acidic than gastrins from most other species. In all, two amino acid substitutions were found in two different locations in the horse G 17 molecule when compared with human G 17. Three more substitutions were seen when the G 34 sequences in the two species were compared.

Following separation of the antral extracts, a large battery of antisera was used. These antisera have been developed using human gastrin as antigen (Rehfeld, Stadil & Rubin, 1972) and they all bound horse gastrin poorly. The differences in structure between the human and horse gastrins probably explains the poor binding of horse gastrin to the antibodies used.

Lack of sulphated gastrins in horses (Paper I)

Studies in other species show that the degree of gastrin sulphation varies widely, from 24 % in the dog antrum (Andersen, 1985) to more than 95 % in the kangaroo (Johnsen & Shulkes, 1993). The present results show that horse gastrin is essentially not sulphated.

According to a consensus rule for sulphation, an acidic residue is required immediately N-terminal to Tyr (Huttner & Baeuerle, 1988). The Ala residues N-terminal to Tyr in horse gastrin seem to be an exception to this rule, possibly compensated for by the preceding stretch of Glu:s. In horses, the net acidity of this particular region is lower than that in other mammalian species and this might therefore help to explain why no sulphated gastrin was observed.

To investigate if the absence of sulphation was due to the peptide structure or to the cellular feature, the horse gastrin cDNA was expressed in HIT-cells. In this cell system, a large amount of sulphated gastrin was expressed, comparable to the amounts obtained from human gastrin. The lack of sulphation in equine gastrin cells may therefore be due to the presence of another cell-type in horses, not expressing the same enzyme distribution as the human gastrin cells. Our results seem to be in conflict with those of Young & Smyth (1990), who reported the existence of sulphated gastrin in the horse. When separating G 34 by anion exchange chromatography, they found two peaks, which they interpreted as showing the presence of sulphated and non-sulphated gastrins. However, artefactual oxidation of Met (to the sulphoxide) represents a possible pitfall, since peptides containing met-sulphoxide elute significantly earlier in anion exchange chromatography than its reduced counterparts. In addition, horse gastrin in their experiments was not measured with antibodies that could distinguish between sulphated and non-sulphated gastrins. Thus, Young & Smyth did not have the opportunity to get immunological support for their observation.

Molecular forms of horse gastrin (Paper I)

It is a common feature in all species that gastrin in tissues, as well as in blood, appears in several molecular forms. Of those molecules, G 17 and G 34 are present in the highest concentrations. However, the quantitative distribution of the two molecules varies within the same animal depending on whether the gastrin has been extracted from the antrum or from the duodenum. The distribution also varies between species. In pig antral tissue the relation between G 17 and G 34 is 91:9. In human antral extracts as much as 60 % of the total gastrin immunoreactivity has been shown to be G 34 (Berson & Yalow, 1971). Similarly, in horses a relatively high proportion of G 34 was found in the antral tissue. Young & Smyth (1990) found even higher proportions of G 34 (32-88 %). Their observation of wide variations in G 34 concentrations in the horse antrum compares favourably with other observations of ours (Sandin & Nilsson, to be published). The relatively high proportion of G 34 indicates a decreased processing at the dibasic cleavage site, Lys77-Lys78, in horses. This may be due to the dependence of the processing enzyme on the structure that is located C-terminally to the cleavage point and possibly also to the sulphation status.

In some species, for example pig and man, gastrin-like immunoreactivity indicating molecules of larger molecular size than G 34 has been demonstrated (Rehfeld, Stadil & Vikelsœ, 1974; Yalow & Wu, 1973; Rehfeld & Johnsen, 1993). In the present study no gastrin-like immunoreactivity indicating larger gastrin molecules was found in the horse. Considering the low affinity between the antibodies used and horse gastrin, the lack of immunoreactivity is not surprising. However, in other gastrin studies in our laboratory (Sandin & Nilsson, to be published) the presence of a larger gastrin molecule

has been indicated. The molecular size of such a molecule can only be speculated upon. Hypothetically, there are three monobasic cleavage sites where such a molecule having a free C-terminal end could be separated. Monobasic sites have previously been demonstrated as possible cleavage sites (Schwartz, 1986). Of these monobasic sites, a cleavage at the arginine site seems most likely, as previously demonstrated in humans (Rehfeld & Johnsen, 1993).

Molecular size and cDNA sequence of horse gastrin (Paper I)

Both the calculated and the measured molecular masses of G 17 were found to be 2039 dalton, whereas the corresponding values for the G 34 molecule were 3812 and 3814 dalton, respectively.

In other investigations, horse gastrin cDNA was determined, which permitted the deduction of a preprogastrin sequence containing 107 amino acids. This sequence contains three more amino acids than are usually found in other species (Walsh, 1994).

Release of gastrin following meal stimulation (Paper II)

It has been shown in other species that, during a meal, gastrin is first released by nervous excitation and when the food enters the stomach the release is further increased (Nilsson *et al.*, 1972) by mechanical (Debas *et al.*, 1974) and chemical (Elwin, 1974) stimulation of the gastric antrum.

In horses, very little is known about the physiological mechanisms regulating gastrin release. The present study was undertaken in an attempt to elucidate the mechanisms responsible for such release. It should be emphasised that demonstration of nervous release of gastrin would require sham feeding experiments on horses provided with a fistulated transplanted oesophagus, as has previously been done in dogs (Nilsson *et al.*, 1972). In the absence of horses prepared in this way, gastrin release has therefore been studied following feeding of different sorts of meals. Stimulation was carried out with three types of meal, differing in composition and volume.

In one experiment, the horses were given an unlimited amount of hay (8-9 kg per horse). In another experiment, hay (0.6 kg/100 kg bwt) was given in combination with grain (0.2 kg/100 kg bwt) and in a third experiment, a small meal consisting of grain (0.2 kg/100 kg bwt) was fed.

Following feeding with grain alone, no immediate release of gastrin was noted. However, there was a gradual increase in gastrin levels, which remained elevated for a long period of time. In dogs, nervous excitation by sham feeding initiates an immediate increase in the plasma gastrin concentration, which becomes further pronounced when the food enters the stomach (Nilsson *et al.*, 1972). The grain meal was eaten fast and the horses had a great appetite. It is therefore reasonable to assume that it caused a strong stimulation of the sensoric senses that are involved in the nervous excitation in other species. The lack of an initial and rapid gastrin response following the small grain meal may

therefore mean that nervous excitation is of minor importance in causing gastrin release in the horse.

When the horses were given a more voluminous hay meal, gastrin release was more rapid than with the small grain meals. In our view, this rapid increase in plasma gastrin concentrations may be due to local gastric stimulation exerted by antral distention. That such stimulation was not seen in the grain experiments may possibly be ascribed to the small volume of the grain meal. The role of mechanical stimulation in causing gastrin release has been shown in another study. Schusser & Obermayer-Pietsch (1992) found increased plasma gastrin concentrations in horses presented for colic (abdominal pain). They also found a significant correlation between the degree of gastric distention and the plasma level of gastrin in these horses.

The duration of the gastrin response varied with the different feeding models. Thus, hay *ad libitum* produced a high and sustained plasma gastrin concentration. When a smaller amount of hay was combined with grain, plasma gastrin concentrations returned to the prestimulation level after approximately four hours. Grain alone seemed to produce a somewhat longer increase in the plasma gastrin concentrations than did the combination with hay. This difference may be explained by the fact that gastric emptying occurs faster when the horses are fed hay than following a grain meal (Meyer, Ahlswede & Pferdekamp, 1980). Grain remaining in the stomach may therefore have caused a more sustained gastrin stimulation by chemical excitation of the gastrin cell. When stimulation of gastrin release was repeated 6 hours after the first stimulation, a similar release pattern was observed.

Gastrin release following stimulation with hay, grain or a mixture of hay and grain has been reported previously (Smyth, Young & Hammond, 1988). The purpose of that study was to compare the effects on insulin and gastrin secretions produced by various feeds and following feeding for different periods of time, rather than to study the mechanisms behind gastrin release. In that study (Smyth, Young & Hammond, 1988), pellets, but not hay, significantly increased plasma gastrin concentrations. The lack of stimulation following hay feeding in their study is difficult to explain.

In summary, the present results suggest that nervous stimulation of gastrin release may play a minor role in horses, whereas both mechanical and chemical stimulation of gastrin secretion seem to occur in the horse.

Gastrin and cortisol release under supposed stress conditions (Paper II)

In man, there are indications that gastrin may act as a stress hormone. Thus, considerable increases in plasma gastrin concentrations have been observed in association with painful venepunctures (Nilsson, personal communication). Elevated plasma gastrin concentrations have also been reported in man following exhausting exercise (MacLaren *et al.*, 1995).

In the present study, no significant effects on plasma gastrin concentrations were seen in association with the insertion of an intravenous jugular catheter, even when the horses seemed to experience pain.

The horses were also subjected to a 24 hour fasting period and to two different treadmill tests to simulate stressful situations. It should, however, be emphasised that it may be difficult to determine whether an animal in a certain situation experiences stress in the sense of something unpleasant.

However, no increases in plasma gastrin concentrations were noted following fasting, although the animals presented strong evidences of being in an irritated and stressful condition. In rats (Lichtenberger, Lechago & Johnson, 1975; Johnson & Guthrie, 1983) and humans (Uvnäs-Wallensten & Palmblad, 1980) prolonged fasting has lowered the plasma gastrin concentrations. In the present experiments, plasma cortisol concentrations remained at a significantly higher level in fasting animals than in animals that had free access to hay.

As in fasting experiments, plasma gastrin concentrations were not increased following exercise. This is in contrast to the previous reported data, finding increased plasma gastrin concentrations in man (MacLaren *et al.*, 1995). Even if the intensity of the exercise may have been similar in the two studies, it should be emphasised that the exercise in horses was performed during 30 min, whereas the participants in the human study ran for 90 min. Thus, exercise length may play an important part in order to increase the plasma gastrin concentrations.

Plasma cortisol concentrations, on the other hand, increased significantly in particular when the horses ran for a longer period of time (30 min). However, increased plasma cortisol concentrations seen in fasting as well as in exercise experiments, may not necessarily reflect stress in the sense of something unpleasant, but may rather be an elevation related to physiological adaptation to a situation where the animals enter a catabolic state.

Pitfalls in studying gastric secretion in cannulated animals (Papers III and IV)

Studies on acid secretion in animals with a gastric cannula are associated with certain pitfalls. The collected gastric juice may be contaminated with regurgitated alkaline duodenal fluid which artefactually increase gastric juice pH and decrease titrated acidity. In horse experiments, an attempt was made to estimate the amount of material regurgitated from the duodenum by measuring the amount of 3α-hydroxy bile acids in the horse gastric juice under basal conditions and following stimulation by different means. A certain amount of bile acids was found in the basal gastric juice. However, the amount decreased dramatically when gastric secretion was stimulated. Therefore, in our view, contamination with regurgitated juice from the duodenum did not significantly influence the results in this study.

Swallowed saliva may also disturb the determination of the acid output, in particular when the stimulation used also activates the secretion of saliva. In

addition, losses of gastric juice through the pylorus may also influence the results. A more optimal design of gastric secretion studies in horses would therefore require isolated gastric pouches of the Heidenhain or Pavlov type.

Effects of HoG17, HuG17, pentagastrin and histamine on acid secretion in horses, dogs and rats (Paper III)

These studies were undertaken to study the rate of basal acid secretion and to elucidate the influence of gastrin and histamine on horses provided with a gastric cannula. Following isolation and determination of the structure of horse G 17 and G 34, the heptadecapeptide gastrin was synthesised. Its biological effect was then determined in horses by infusing increasing doses of gastrin every 45 min. For comparison, horses were also stimulated with pentagastrin and histamine in a similar way. For additional comparison, HoG17, HuG17, pentagastrin and histamine were also given to dogs and rats, which were similarly provided with a gastric cannula for collection of the gastric juice.

In the horse and dog experiments, HoG17 produced a maximal acid secretion of similar magnitude to that produced by pentagastrin. The maximal acid secretion produced by histamine was somewhat lower in horses but somewhat higher in dogs compared to the other stimulants. HuG17 was slightly more effective than HoG17 in stimulating maximal acid secretion in dogs. In rats, HoG17 and histamine produced similar maximal acid outputs, whereas the maximal secretions stimulated by HuG17 and pentagastrin were somewhat (20 %) higher.

The results related above indicate that the synthetic horse gastrin was quite effective in stimulating gastric acid secretion in all three species investigated. The somewhat lower maximal acid output in rats in comparison with secretion induced by human G 17 may be due to the fact that rat gastrin differs from horse gastrin by 5 amino acids, whereas human gastrin differs from rat gastrin by only 3 amino acids.

In horses, TAO was somewhat lower following histamine stimulation than the responses produced by horse gastrin and pentagastrin. The lower histamine-stimulated acid secretion could be due to the concomitant infusion of an H_1-receptor antagonist, given in order to protect the horses from vascular and respiratory side effects that may be caused by higher doses of histamine (Aguilera-Tejéro, Pascoe & Woliner, 1993). A lower maximal acid response in horses has also been reported elsewhere, when an H_1-receptor antagonist was administered together with pentagastrin (Kitchen, 1997; Kitchen, Merritt & Burrow, 1998).

However, the most striking difference between the species was found when the doses of histamine needed to cause maximal acid stimulation were compared. Thus, maximal secretion in horses only required 16 μg/kg bwt/h of histamine, a dose that hardly produces any secretion in the rat. The dose needed to produce maximal effect in dogs was 64 μg/kg bwt/h and that in rats as much

as 4000 µg/kg bwt/h was needed. Horses thus required 250-500 times less histamine than the rats to produce maximal acid secretion. The sensitivity to histamine stimulation seems to be related to the number of ECL-cells present in the acid secreting portion of the stomach. Thus, plenty of ECL cells can be found in the rat stomach (Håkanson *et al.*, 1986), whereas fewer cells are seen in dogs (Håkanson *et al.*, 1986) and horses (Sundler[1], personal communication).

Effects of nervous excitation on acid secretion in horses (Paper IV)

As mentioned in the introduction, nervous excitation of gastric acid secretion may be produced by different means. In the present study nervous activation was induced by insulin hypoglycaemia, a cholinergic agonist and teasing. In addition, animals were given atropine, which in lower doses counteracts the muscarinic effect at the parietal cell level.

Insulin-induced hypoglycaemia is an established way to stimulate secretion of acid from the stomach and its effect has been demonstrated in several species. The stimulation of the parietal cells is supposed to be mediated by cholinergic fibres in the vagal nerves (La Barre & Cespèdés, 1931). In man, insulin-induced hypoglycaemia and determination of the gastric acid output has been a tool in determining whether a surgical vagotomy has been complete or not (Hollander, 1946; Bachrach, 1962).

When insulin hypoglycaemia was induced in the horses, inhibition of acid secretion and reduction of the secreted volume were found instead of stimulation. Such an effect has not been demonstrated previously in mammals, but in hens (Ruoff & Sewing, 1972), which also have a basal gastric secretion. In animals in which the secretion of acid has been stimulated, on the other hand, different modes of nervous stimulation have been shown to inhibit gastric acid secretion. Thus, insulin-induced hypoglycaemia and 2-deoxy-D-glucose (Spencer & Grossman, 1971) and sham feeding (Sjödin, 1975; Sjödin & Andersson, 1977) inhibit pentagastrin-induced acid secretion in dogs, whereas secretion stimulated with histamine is less influenced.

Since the horse and the hen have a basal secretion of acid, their secretion could be considered to be stimulated, even if the mechanisms responsible for such stimulation are unknown. If such an assumption is made, the inhibitory effect of insulin-induced hypoglycaemia in the present experiments is not too surprising. However, it remains to be explained how this inhibition is accomplished. Hypothetically, the inhibition may be mediated by vagal fibres acting directly on the parietal cells or indirectly via the enteric nervous system or via the endocrine cells in the corpus portion of the stomach. Such endocrine cells may act in a paracrine way on the parietal cells. Inhibition may also be exerted by nervous release of humoral substances acting in an endocrine mode.

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At present, the inhibition induced by insulin hypoglycaemia in this study cannot be ascribed to any particular mechanism. A number of substances that inhibit gastric acid secretion have been demonstrated in vagal fibres. For example, immunoreactivity of somatostatin (Lundberg *et al.*, 1978; Uvnäs-Wallensten, Efendic & Luft, 1978), VIP (Said & Rosenberg, 1976), gastrin/CCK (Uvnäs-Wallensten *et al.*, 1977; Lundberg *et al.*, 1978) and CGRP (Clague, Sternini & Brecha, 1985) are present in vagal nerves. In addition, it has been shown that somatostatin (Konturek *et al.*, 1976), CGRP (Taché, Raybould & Wei, 1991), Gastrin/CCK (Gillespie & Grossman, 1963) and VIP (Villar *et al.*, 1976) inhibit gastric acid secretion. A common feature of VIP (Chiba *et al.*, 1980), CCK-like peptides (Bengtsson, Lundqvist & Nilsson, 1989) and CGRP is that they may all cause release of somatostatin from D-cells in the gastric mucosa. Hypothetically, vagal stimulation may also release peptides inhibiting acid secretion. For example, it has been demonstrated that the intestinal hormones secretin and CCK (Gillespie & Grossman, 1964) and the putative inhibitor bulbogastrone (Andersson & Uvnäs, 1961; Nilsson, 1980*a*) from the bulbar mucosa inhibit gastric acid secretion.

However, there may also be another explanation for the inhibition following insulin-induced hypoglycaemia. As a consequence of the hypoglycaemia, impulses will arise in the brain that causes release of epinephrine from the adrenals. Epinephrine reduces gastric acid secretion in dogs (Hovendal *et al.*, 1981) but increases it in rats (Canfield & Price, 1984). The effect of epinephrine on gastric acid secretion in horses is not known and, in particular, it is not known whether the amounts of epinephrine released in association with insulin-induced hypoglycaemia are sufficient to influence the gastric acid output.

In order to find out whether gastric acid secretion is under cholinergic influence, two series of experiments were carried out. In one series, the horses were given a muscarinic receptor agonist, bethanechol, and in another atropine.

Stimulation with bethanechol produced a considerable increase in the volume collected from the gastric cannula, but also a considerable reduction of its acidity. The increase in pH was most likely due to swallowed saliva, since the bethanechol also produced a copious salivary secretion. Attempts were therefore made to estimate the buffering capacity of saliva collected from the mouth and the amounts of saliva necessary to raise the pH during basal acid secretion (3.1) to the pH determined after stimulation. According to these calculations, the collected volume of 1060 ml following stimulation required 125 ml of saliva to raise the pH to the observed level in that sample. Such an amount of saliva was no doubt swallowed by the horse during a 15 min period. It therefore seems reasonable to assume that bethanechol stimulated the secretion of acid from the stomach, but that this secretion was neutralised by the swallowed saliva.

When the horses were given the cholinergic antagonist atropine, basal acid secretion was abolished. The results from the present study together with results from *in vitro* experiments with isolated parietal cells (Campbell-

Thompson, 1994) demonstrating increased aminopyrine uptake following carbachol stimulation, thus support the concept that cholinergic stimulation also takes part in the activation of the parietal cells in horses.

Sham feeding evokes nervous stimulation of both gastrin and acid secretion (Nilsson *et al.*, 1972; Feldman & Richardson, 1986). Teasing constitutes a considerably weaker form of such stimulation which results in lower secretory responses of gastrin (Feldman & Richardson, 1986) and acid (Schapiro *et al.*, 1967). In the present study, horses were exposed to a bucket of grain for 15 min. This exposure resulted in a certain (31 %), but not statistically significant, increase in the acid output.

Effects of nervous excitation, HoG17, HuG17, pentagastrin and histamine on gastric juice volume (Papers III and IV)

As mentioned earlier, there is a continuous basal secretion of gastric juice in the horse. When atropine was administered to the horses, gastric secretion was inhibited, indicating a cholinergic stimulation of the basal secretion. Insulin-induced hypoglycaemia also significantly reduced the basal secretion. Together, these observations suggest that basal secretion of gastric juice in the horse is under vagal influences and that the cholinergic impulses contribute to its stimulation.

The two heptadecapeptide gastrins and pentagastrin increased the gastric juice volume in a dose-dependent manner in all species tested. In the histamine experiments, an H_1-receptor antagonist, pyrilamine maleate, was administered together with histamine in order to reduce the side effects produced by histamine. Hypothetically, the secreted gastric juice volume may have been reduced in these experiments, since histamine may be a less potent stimulant of volume secretion than gastrin. Another explanation might be that H_1-receptors play a role in the stimulation of volume secretion in the stomach, and administration of an H_1-receptor antagonist causes a reduction in secretion. A further possibility is that the H_1-receptor antagonist may have a certain cross-reactivity with the H_2-receptor, resulting in decreased gastric acid secretion. The results in this study are similar to a previous report by Kitchen (1997). In that study pyrilamine maleate given together with pentagastrin caused a reduction in gastric acid output. The authors postulated that the lower volume collected may have been caused by a reduction in stomach blood flow.

In rats and dogs, pentagastrin and HuG17 produced similar maximal volumes, while HoG17 evoked maximal volumes that were somewhat lower. Histamine was most potent in stimulating the volume secretion in dogs, whereas the histamine-stimulated secretion in rats essentially equalled that produced by pentagastrin and HuG17.

Conclusions

1. Determination of the cDNA indicates that horse preprogastrin contains three amino acids more than preprogastrins from other species.

2. The amino acid sequences of horse gastrin G 17 and G 34 differ from those of other species in several respects. In particular, horse gastrin contains a Lys instead of a Glu in the acidic stretch of amino acids preceding the bioactive C-terminal portion of the molecule, which renders the molecule less acidic than gastrins of other species. This may explain why horse gastrins do not bind well to antibodies produced with human gastrin as antigen.

3. Horses do not express sulphated gastrins and probably lack the required enzyme system.

4. In comparison with other species, a relatively large amount of G 34 is found in the horse antrum.

5. Nervous excitation seems to play a minor role in stimulating gastrin release in horses. Following local stimulation of the gastric antrum by a meal, a significant increase in the plasma gastrin concentration is found. A grain meal produces a more prolonged gastrin release than a hay meal, possible due to the prolonged emptying of the grain meal from the stomach.

6. The synthesised horse gastrin 17 effectively stimulates gastric acid output in horses, dogs and rats.

7. Horses are extremely sensitive to histamine stimulation. Thus, dogs require 4-8 times more and rats 250-500 times more histamine to produce maximal acid secretion.

8. Nervous stimulation by teasing evokes a small and short lasting but not statistically significant acid stimulation in horses.

9. Insulin-induced hypoglycaemia reduces the basal acid output in the horse, which is in contrast to the effect seen in most other species.

10. Cholinergic excitation takes part in the basal acid and volume secretion in the horse, since atropinization results in total inhibition of the basal output.

Svensk sammanfattning

Undersökningar från senare år visar att hästar mycket ofta har magsår. Lågt pH i magsäcken anses vara en av de främsta anledningarna bakom uppkomsten av sår. Mycket litet är känt om de mekanismer som styr magsäckens sekretion av syra hos hästen. Den föreliggande avhandlingen avser därför studier av gastrin- och syrasekretionen hos häst.

Från antral vävnad insamlad på slakthus, har två av hästens gastriner, G 17 och G 34, med 17 respektive 34 aminosyror renats och strukturbestämts. Vidare har cDNA-sekvensen för gastrinets prepropeptid bestämts. Hästgastrinet skiljer sig i ett flertal avseenden från andra arters gastrin, främst genom att den vanligen förekommande långa glutaminsyrasekvensen hos hästens gastrin är avbruten med en basisk aminosyra (lysin). Härigenom blir hästgastrinet mindre surt till sin karaktär. Som enda hittills kända djurart saknar hästen sulfaterat gastrin. Detta beror på att hästens gastrinceller saknar förmåga till sulfatering.

Med radioimmunologisk teknik har gastrinfrisättningen studerats hos hästar vid utfodring med kraftfoder, hö eller en kombination av dessa två foder. De olika utfodringsförsöken ger upphov till sekretionskurvor, vilka ger anledning till följande tolkning: Nervös stimulering, som hos exempelvis hund synes spela en stor roll för frisättningen av gastrin, förefaller vara av mindre betydelse hos hästen. Huvudparten av gastrinfrisättningen sker i stället genom att födan lokalt aktiverar magsäckens gastrinfrisättande mekanismer. Detta kan ske genom mekanisk eller kemisk inverkan på antrumslemhinnan.

Undersökningar har även utförts i syfte att klarlägga om gastrinet fungerar som ett stresshormon hos hästen i samband med smärta, fasta och fysisk ansträngning. Det undersöktes bland annat hur löpning på rullmatta påverkar plasmakoncentrationen av gastrin och cortisol. Ingen stegring av gastrinkoncentrationen noterades, men en viss ökning av koncentrationen av cortisol. Cortisolstegringen antas bero på en metabolisk omställning orsakad av det fysiska arbetet snarare än av stress.

Syrasekretionsförsöken i detta arbete har utförts på djur försedda med en ventrikelkanyl. Efter syntes av hästens G 17-peptid har peptidens effekter studerats på hästens syrasekretion och effekterna jämförts med den stimulerande effekten av gastrinets pentapeptid (Peptavlon®) och av histamin. Som jämförelse har den syrastimulerande effekten också studerats på hund och råtta. På de två senare arterna har även humant G 17 använts. Resultaten visar att den syntetiserade gastrinpeptiden effektivt stimulerar såväl sekretionen av syra som volymflödet från magsäcken hos samtliga tre arter. Hos hund stimulerar hästpeptiden syrasekretionen lika effektivt som humanpeptiden medan sekretionen hos råtta blir något lägre av hästpeptiden. Den senare skillnaden kan bero på att det förekommer större strukturella skillnader mellan råttans och hästens gastrin än mellan råttans och människans. Hos hästen är histaminet ett svagare stimulus av magsäckens volymssekretion.

Försök har även gjorts att studera nervösa faktorers inverkan på hästens syrasekretion. Försöken har utförts på hästar försedda med en magsäckskanyl. Sekretionen har påverkats genom

1. teasing, varvid djuren får se och känna lukten av födan men inte äta den
2. insulin-inducerad hypoglykemi
3. en kolinergisk agonist, bethanechol, och
4. ett antikolinergikum, atropinsulfat

Teasing gav upphov till en obetydlig sekretionsökning medan insulinhypoglykemin, till skillnad från vad som brukar vara fallet hos de flesta andra arter, hämmade hästens basala syrasekretion. Analys av sekretionssvaret efter bethanecholtillförseln försvårades av den kraftiga salivsekretion som uppstod, vilken delvis svaldes av hästen och blandades med magsaftssekretionen, varigenom pH höjdes kraftigt. En efterföljande analys talade emellertid för att bethanechol framkallat en stimulering av syrasekretionen. Atropinisering hämmade helt hästens basala syrasekretion.

Resultaten talar för att nervös kolinergisk stimulering aktiverar den basala syrasekretionen hos hästen. Det föreslås att insulinhypoglykemin aktiverar nervösa mekanismer som verkar hämmande på syrasekretionen genom en direkt effekt på parietalcellerna, indirekt via det enteriska nervsystemet eller via parakrina eller endokrina mekanismer.

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Acknowledegements

I wish to express my sincere gratitude to everyone who have supported me and made this thesis possible and especially to:

Professor Göran Nilsson at the Department of Physiology, Associate professor Göran Björnhag at the Department of Animal Physiology and Professor Arne Lindholm at the Department of Medicine and Surgery, all at the Swedish University of Agricultural Sciences, the Department of Medicine and Surgery, Huddinge University Hospital in Huddinge, Professor Jens Rehfeld at Rigshospitalet in Copenhagen, Danmark, Astra Hässle AB in Gothenburg and Dr Frank Andrews at the Department of Large Animal Clinical Science, Knoxville, Tennessee, USA for letting me use the facilities at their departments.

Professor Göran Nilsson, my main superadvisor and friend for introducing me into the field of gastric physiology. I appreciate the valuable knowledge of science I have learned from you during these years and for the continuous full-hearted support I have experienced concerning all sorts of things ranging from scientific matters to soccer. Moreover, I am deeply grateful for the freedom of independence you have given me.

Dr Frank Andrews my assistant superadvisor and collegue. I am most grateful for inviting me into your research group. I really enjoyed my time in Knoxville and I think the work we did at your department was very fruitful. I will always remember the tough games at the soccerhouse in the evenings.

Dr Anders H. Johnsen for all help with the gastrin characterisation and for teaching me all kinds of methods. I am also grateful for our fruitful discussions about gastrin that we had during my stay at Rigshospitalet.

Professor Jens Rehfeld for letting me be a guest in his laboratory at Rigshospitalet in Copenhagen and giving me the oppurtunity to characterise horse gastrin. I am also grateful for the kind donations of gastrin antibodies which have made parts of my thesis possible.

Professor Kerstin Olsson for always being enthusiastic, offering me valuable criticism and never ending encouragement in my research

Dr Yvonne Ridderstråle for most valuable help with computers and for all advices regarding the construction of my thesis.

Dr Andrzej Madej for advices concerning radioimmunoassays, statistics and for nice and friendly discussions.

Dr Jens Häggström for valuable help and advices with the statistics.

Professor Arne Lindholm and Bo Sjöholm for assisting me with the plasma gastrin study.

Agneta Karlsson at Astra Hässle AB, Annika Wagman at Huddinge University Hospital and Eva Werner at SLU for technical assistance with both gastrin analyses and acid secretion studies. You have been most valuable.

Dr Åke Engström for synthesising the horse gastrin peptide and by that making the third paper possible.

Margareta Topel and Sören Johansson for nice and friendly discussions.

Camilla Jüllig for making the cover layout and changing Soo into a princess.

Åsa, Olga and Lena for taking good care of the rats.

the staffs and fellow PhD-students at the Department of Animal Physiology, SLU and at the Department of Large Animal Clinical Scinece, Knoxville, USA for friendships and good laughs.

my dear friends Anna, Kinfe, Semir, Jon-Olav, Tomas, Maria and Ann

Sessan, Tusse and Vilgot my best friends among cats.

my beloved parents; my father Mårten and my mother Barbro, for always being there for me with support and interest.

Mathias for interesting discussions about everything between heaven and earth but most of all for being the person you are

Finally, but not least, I would like to thank Sara, to who my heart belongs. Thanks for your unconditional love, for your unfailing support and for your encouragement. I hope I can make it up to you in the future.

The financial supports from the Swedish Council for Forestry and Agricultural Research (SJFR), the Swedish Racing Board (ATG), the Tennessee Equine Veterinary Research Organization, the Danish Biotechnology Program for Signal peptides, the Danish Medical Research Council, the Lundbeck, Velux, John and Birthe Meyer, Brdr. Hartman and Novo Nordisk Foundations, "SLU-medel för internationalisering av forskarutbildningen" are gratefully acknowledged.

In memoriam

Dr Stig-Göran Olovsson, a friend and collegue, for supporting my research and introducing me to the equine group in Knoxville. I am greatly indebted to Stig-Göran for his contributions to my thesis work.

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ISSN 1401-6257
ISBN 91-576-5908-7