Acid Transport Through Gastric Mucus

A Study in vivo in Rats and Mice

BY

MIA PHILLIPSON
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Abstract


The gastric mucosa is frequently exposed to endogenously secreted hydrochloric acid of high acidity. Gastric mucosal defense mechanisms are arranged at different levels of the gastric mucosa and must work in unison to maintain its integrity.

In this thesis, several mechanisms underlying gastric mucosal resistance to strong acid were investigated in anesthetized rats and mice. The main findings were as follows:

1. Only when acid secretion occurred did the pH gradient in the mucus gel withstand back-diffusion of luminal acid (100 mM or 155 mM HCl), and keep the juxtamucosal pH (pHm) neutral. Thus, when no acid secretion occurred and the luminal pH was 0.8-1, the pH gradient was destroyed.
2. Bicarbonate ions, produced concomitant with hydrogen ions in the parietal cells during acid secretion and blood-borne to the surface epithelium, were carried transepithelially through a DIDS-sensitive transport.
3. Prostaglandin-dependent bicarbonate secretion seemed to be less important in maintaining a neutral pHm.
4. Removal of the loosely adherent mucus layer did not influence the maintenance of the pHm. Hence, only the firmly adherent mucus gel layer, approximately 80μm thick, seemed to be important for the pHm.
5. Staining of the mucus gel with a pH-sensitive dye revealed that secreted acid penetrated the mucus gel from the crypt openings toward the gastric lumen only in restricted paths (channels). One crypt opening was attached to one channel, and the channel was irreversibly formed during acid secretion.
6. Gastric mucosal blood flow increased on application of strong luminal acid (155 mM HCl). This acid-induced hyperemia involved the inducible but not the neural isoform of nitric oxide synthase. These results suggest a novel role for iNOS in gastric mucosal protection and indicate that iNOS is constitutively expressed in the gastric mucosa.
7. It is concluded that a pH gradient in the gastric mucus gel can be maintained during ongoing acid secretion, since the acid penetrates the mucus only in restricted channels and bicarbonate is carried from the blood to the lumen via a DIDS-sensitive transporter.

Keywords: gastric acid, pH-sensitive microelectrode, mucus, mucosal blood flow, nitric oxide, intravital microscopy, laser Doppler flowmetry

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III The importance of mucus layers and bicarbonate transport in preservation of gastric juxtamucosal pH.  
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IV Inducible Nitric Oxide Synthase is involved in acid induced gastric hyperemia in rats and mice.  
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Abbreviations

bw  body weight
b.g.  by gavage
$^{51}\text{Cr-EDTA}$  $^{51}$chromium-labeled
ethylenediaminetetraacetate
CGRP  calcitonin gene-related peptide
eNOS  endothelial nitric oxide synthase
ECL cell  enterochromaffin-like cell
ENS  enteric nervous system
G cell  gastrin cell
HCl  hydrochloric acid
iNOS  inducible nitric oxide synthase
i.p.  intraperitoneal
i.v.  intravenous
L-NIL  L-N$^\text{o}$-(1-iminoethyl)-lysine
L-NNA  N$^\text{\textit{n}}$–nitro–L–arginine
LDF  laser Doppler flowmetry
MAP  mean arterial blood pressure
nNOS  neuronal nitric oxide synthase
NO  nitric oxide
NOS  nitric oxide synthase
pH$_{\text{jm}}$  juxtamucosal pH
PCR  polymerase chain reaction
PGE$_2$  prostaglandin E$_2$
RT-PCR  reverse transcriptase PCR
rtRT-PCR  real-time RT-PCR
SMTC  S-methyl-L-thiocitrulline
Introduction

The gastric epithelium is regularly exposed to the endogenous aggressive factors acid and degrading enzymes such as pepsin. How the stomach can resist autodigestion, i.e., remain safe and sound when forming a harmful environment for bacteria and inducing enzymatic degradation of proteins, has intrigued physiologists for centuries. Dr Bernard wrote in 1856 that the mucus coating the stomach rendered it impermeable to the acidic gastric juice, as if it were a porcelain vase (Bernard 1856, LaMont 2000). The question of how it achieves this protective effect has remained unanswered until recently.

In addition to the harsh intrinsic environment, the stomach also faces ingested bacteria, food and drink and sometimes also ulcerogenic drugs, and has to function as a barrier to protect the interior from the external environment.

Acid secretion and regulation

Hydrochloric acid (HCl) is produced and secreted by the parietal cells located in the gastric glands in the corpus part of the stomach. The acid activates the proteolytic enzyme pepsin, creates a bacteriotoxic environment, and initiate degradation of proteins. Histamine, gastrin and acetylcholine stimulate acid secretion and are reported to potentiate the effects of one another (Helander 1988). Histamine is secreted by the enterochromaffin-like (ECL) cells that are present in the oxyntic glands and diffuses to the H2 receptors located on the basolateral membrane of the parietal cells. Gastrin is produced by the gastrin (G) cells in the antrum, and is transported to the corpus in the systemic circulation. Gastrin stimulates acid secretion mainly by stimulating the ECL cells to secrete histamine, although gastrin receptors (cholecystokininbb, CCKbb) have also been found on the parietal cells (Kopin et al. 1992). Acetylcholine is released from the vagal nerves and activates G cells, ECL cells and parietal cells, but its efficacy in stimulating acid
secretion varies greatly between species and this effect in humans is very weak. H₂-receptor antagonists (for example ranitidine, used in study III) inhibit acid secretion, indicating that histamine plays a key role in the stimulation of acid secretion.

When the parietal cells are activated to produce acid, intracellular tubulovesicles are translocated to the apical membrane, thereby increasing the area of the secretory membrane, and the transporter H⁺/K⁺-ATPase is activated. Omeprazole (used in study II) is an example of an inhibitor of the H⁺/K⁺-ATPase (Helander 1988). When stimulated, the parietal cell can secrete an acid with a concentration of 155 mM, i.e., with a pH of ≈0.8 (Niv et al. 2002). For every hydrogen ion formed, one bicarbonate ion is created through the enzyme carbonic anhydrase. To prevent alkalinization of the parietal cell during acid secretion, basolateral extrusion of bicarbonate is important. This occurs mainly through the Cl⁻/HCO₃⁻ exchanger. The bicarbonate enters the bloodstream and can be measured as an alkaline tide in arterial blood and in urine after a meal or histamine stimulation of acid secretion (Rune 1965, Niv et al. 2002).

To exert its function in the gastric lumen, the acid must pass through the mucus layer (fig. 1). How this occurs without acidification of the mucus layer itself has not previously been established. The hydrostatic pressure that has recently been measured in the lumina of rat gastric glands might be the driving force for acid transport through the mucus layer (Holm et al. 1992). This pressure increases from approximately 12 to 17 mmHg on stimulation of acid secretion.

![Figure 1](image_url)

Figure 1.
Schematic drawing of a cross-section of the gastric corpus mucosa with adhering mucus layer and blood supply. N.B. acid must pass through the mucus to the gastric lumen.
Gastric mucosal defense

The defense mechanisms of the gastric mucosa are crucial for the maintenance of an effective barrier and for preventing the stomach from digesting itself. The defense is arranged at different levels, which work in concert for effective protection.

The preepithelial level or the first line of defense consists of the mucus layer and bicarbonate secreted into the mucus, creating a pH gradient within the mucus.

The epithelial level consists of intercellular tight junctions and proton and bicarbonate transport systems.

The postepithelial level consists mainly of an effective blood flow and the gastrointestinal autonomic nervous system, the enteric nervous system (ENS).

The preepithelial level

The mucus layer forms a continuous coat over the gastric epithelium. Bicarbonate is secreted from the epithelium into the mucus layer, where it neutralizes acid that is back-diffused from the lumen of the stomach and forms a pH gradient, with a higher pH at the epithelial cell surface (Ross et al. 1981, Schade et al. 1994).

The mucus layer

A continuous layer of mucus gel, secreted by the surface epithelial mucous cells and the mucous neck cells, covers the gastric mucosa. The mucus gel serves as a physical barrier and molecules with the size of pepsin cannot penetrate through diffusion. However, hydrogen ions are able to diffuse through the gel, although their diffusion is approximately four times slower than that through an unstirred layer of equivalent solution (Williams et al. 1980).

The mucus consists of 5% high molecular weight glycoproteins (10^6, Allen 1989), called mucins, and 95% water together with electrolytes and small
amounts of lipids and proteins, including immunoglobulins. The glycoproteins are the gel-forming components of the mucus and consist of a protein core that has highly glycosylated regions. The carbohydrate side chains bound to the protein vary in structure and might influence the physical properties of the mucin. The nonglycosylated regions of the protein core are rich in cysteine and can form inter-mucin disulfide bridges, which create the polymeric structure of the gel by linking the mucins together.

The surface mucous cells secrete mucins of the MUC5AC type and the mucous neck cells secrete MUC6 mucins. Whether the different mucins exhibit different physical behaviors and physiological functions is still unknown. It has recently been found possible to separate the mucus layer covering and adherent to the corpus mucosa into two different layers, in addition to the loose mucus in the gastric lumen (Atuma et al. 2001). The most luminal of the two layers, the loosely adherent mucus, can be removed by suction or by rubbing with a cotton tip, while the inner layer, the firmly adherent mucus, cannot be removed by this physical means. The physical properties and physiological importance of the different layers are unknown, as is their composition, and it is not known whether they differ in permeability to acid.

The thickness of the mucus layers depends on the secretion of mucins and the degree of erosion and proteolytic degradation of the layers. Mucus secretion is stimulated by agents such as prostaglandins and nitric oxide, whereas the mucus layer is degraded by pepsin (Allen et al. 1993, Brown et al. 1992).

**Bicarbonate secretion**

The surface epithelial cells secrete bicarbonate into the mucus gel. The bicarbonate neutralizes back-diffused acid and creates a pH gradient in the mucus layer, with a neutral pH at the cell surface when the luminal pH is low. Bicarbonate can be produced from carbon dioxide and water in the gastric mucosal surface epithelial cells by the enzyme carbonic anhydrase (Allen et al. 1993). Furthermore, Teorell (1951) demonstrated that for each proton secreted from the parietal cell, one bicarbonate ion is released from the basolateral membrane of the parietal cell to the capillaries of the mucosa. The capillaries are arranged along the gastric glands and are directed toward the surface epithelium. Thus, during acid secretion, bicarbonate will be transported by the blood to the surface epithelium, where it will be available for transport across the surface epithelial cells into the mucus.
Vagal stimulation (Forssell et al. 1985), prostaglandins (Flemström 1994, Forssell et al. 1988, Garner et al. 1979, Rees et al. 1984, Takeuchi et al. 1997), gastric distension, and acid in the gastric lumen all increase gastric bicarbonate secretion. Experiments in vitro have shown that gastric bicarbonate secretion is dependent on luminal chloride ions, indicating the presence of an apical Cl⁻/HCO₃⁻ exchanger (Flemström 1980). However, the route by which bicarbonate traverses the surface epithelium during acid secretion has not yet been established.

**pH gradient in the mucus gel**

A millionfold proton concentration gradient can exist between the gastric lumen and the blood, and pH gradients have been found in the mucus layer covering the gastric mucosa by means of inserted pH-sensitive electrodes (Ross et al. 1981, Schade et al. 1994). The gradient succeeds in keeping the epithelial surface neutral (juxtamucosal pH, pH jm) while the lumen pH is 2 in both acid-secreting and non-secreting mucosae (Schade et al. 1994).

Naturally, bicarbonate secretion is necessary for the creation of a pH gradient. Moreover, the mucus layer is crucial for the existence of a pH gradient, since it consists of an unstirred layer in which neutralization of back-diffused acid by secreted bicarbonate can occur. In the Necturus antrum, a luminal mucus layer was shown to be necessary for keeping the juxtamucosal and intracellular pH neutral in the presence of luminal acid (Kiviluoto et al. 1993). The existence of a pH gradient in the gastric mucus during acid secretion is a physiological paradox, since acid, secreted into the gastric glands, has to pass through the mucus gel to reach the gastric lumen.

**The epithelial level**

**Gastric mucosal permeability and cellular transporters**

The mucosa of the gastric corpus is a tight epithelium and under normal conditions it is relatively impermeable to transport of luminal contents, including water. The apical cell membrane of gastric surface epithelial cells has a low permeability to hydrogen ions (Ashley et al. 1987, Kivilaakso et al. 1988), and the tight junctions connecting the surface cells to each other are
even less conductive for ions than is the cellular pathway (Hirst 1989). The paracellular pathway contributes only to approximately 25% of the total tissue conductance. Cellular transporters that transport bicarbonate in a luminal direction and protons toward the buffering blood are important for the viability of the surface cells. Ion transporters located in the gastric surface epithelial cells are illustrated in figure 2. Gastric epithelial cells express the Na⁺/H⁺ exchanger NHE2 basolaterally. This exchanger is activated when the extracellular pH increases, and might be involved in transporting bicarbonate transcellularly (Shull et al. 2000).

![Figure 2. Schematic drawing of the ion transporters in the gastric surface epithelial cell.](image)

**The postepithelial level**

**Gastric mucosal blood flow**

The gastric mucosal blood flow is an important part of the defense, as the circulating blood dilutes, neutralizes and carries away noxious substances that have managed to overcome the more luminal barriers. The blood stream also has an important function in transporting oxygen, nutrients and gastric hormones to the different mucosal cell types.
The mucosal capillaries are arranged along and in close proximity to the gastric glands (Gannon et al. 1982). This architecture is of special relevance for the oxygen-consuming parietal cells and for the bicarbonate transport from the acid-secreting parietal cells to the surface epithelium. At the level of the gastric epithelium, the capillaries form a honeycomb network around the openings of the gastric pits. Hence, the distance between the capillaries and the surface epithelial cells is minimized. Progressing toward the gastric epithelial surface, the capillaries become increasingly fenestrated, which facilitates transport across the capillary membrane. The capillaries empty into collecting mucosal venules oriented perpendicular to the luminal surface.

The gastric mucosal blood flow is regulated at the level of the submucosal arterioles and is under the intricate control of the central and enteric nervous systems, autocrine and paracrine regulation of hormones and growth factors, and mucosal production of eicosanoids. For example, gastric hyperemia caused by mucosal acidification by luminal acid and barrier-breaking substances (e.g., ethanol or sodium taurocholate) is due to activation of sensory afferent nerves, leading to release of calcitonin gene-related peptide (CGRP) in the vicinity of the submucosal arterioles and generation of nitric oxide (NO) (Li et al, 1992). Impairment of this neurally mediated hyperemic response through disruption of the sensory afferent nerves, antagonism of CGRP receptors, or blockade of NO synthesis, results in a significant increase in the susceptibility of the gastric mucosa to damage (Holzer 1998), indicating the importance of a sufficient mucosal blood flow for the preservation of the mucosal barrier.

**Nitric oxide and mucosal blood flow**

Nitric oxide has been reported to influence different components of gastric mucosal defense, such as mucosal blood flow, mucus secretion and mucosal permeability (Wallace et al. 2000). NO is produced together with L-citrulline from the amino acid L-arginine and molecular oxygen under enzymatic catalysis. In mammals, three isoforms of the NO-synthesizing enzyme, nitric oxide synthase (NOS), encoded by different genes, have been identified (Knowles et al. 1994). The constitutively expressed enzyme is Ca$^{2+}$-dependent and can be divided into isoforms associated with neurons (nNOS, type I) and isoforms present in the endothelium lining the vasculature (eNOS, type III). NO produced in the endothelial cells diffuses to the underlying vascular smooth muscle cells, where it stimulates soluble
guanylate cyclase, leading to elevated cGMP levels, and relaxation of the vascular smooth muscle. The inducible NOS (iNOS, type II) is Ca\(^{2+}\)-independent and needs a stimulus (cytokines, lipopolysaccharides) for expression in specific cell types, e.g., macrophages, neutrophils, and endothelial and epithelial cells. It is generally believed that the constitutively expressed isoforms are responsible for the normal physiological effects of NO, whereas iNOS is activated in different pathophysiological states (Wallace et al. 2000).

The gastric mucosal surface cells have been shown to contain a large quantity of NOS that resembles the nNOS isoform (Price et al. 1996). The involvement of this epithelial NOS in gastric mucosal defense has not yet been investigated.
Aims of the investigation

The overall aim of this investigation was to further elucidate the mechanisms underlying gastric mucosal resistance to strong acid. More specifically, the following questions were addressed:

- How is the juxtamucosal pH influenced when the luminal pH decreases to 1?
- Is blood-borne bicarbonate originating from the parietal cells needed for neutralization of back-diffused acid?
- Is prostaglandin-stimulated bicarbonate secretion important for neutralization of back-diffused acid?
- How are bicarbonate ions transported through the gastric surface epithelium?
- How important is the loosely adherent mucus layer for the maintenance of a neutral juxtamucosal pH?
- How does endogenously secreted acid penetrate the mucus gel without disrupting the pH gradient?
- Which NOS isoform is involved in the acid-induced hyperemia?
Materials and methods

Animals

The studies were performed on a total of 174 rats and 44 mice.

Rats: Male Sprague Dawley rats (study I: Möllegaard Breeding Ctr. Ltd., Ejby, Denmark; study III: Charles River, Uppsala, Sweden; study IV: BK, Stockholm, Sweden) and F1 hybrids of Lewis and DA rats (study II: Animal Department at the Biomedical Center, Uppsala, Sweden), weighing between 150 and 290 g, were used.

Mice: Mice weighing between 22 and 41 g were used (study IV). Breeding pairs of mice deficient in iNOS were kindly provided by J.S. Mudgett (Merck Research Laboratories, Rahway, NJ) and J.D. MacMicking and C. Nathan (Cornell University Medical College, New York, NY). The mice (background C57BL/6x129SvEv) were generated by gene targeting in embryonic stem cells as previously described (MacMicking et al. 95). Homozygous iNOS mutants were maintained by interbreeding the F2 generation, and males were used in the experiments. For wild-type controls, male C57BL/6x129Sv were used (Taconic Farms, Germantown, NY).

Breeding pairs of heterozygous nNOS deficient mice were purchased from The Jackson Laboratory, Maine, USA. The mice (background C57BL/6x129Sv) were generated by gene targeting in embryonic stem cells (Huang et al. 1993). The offspring from heterozygote mating were 25% homozygous mutant (-/-), 25% wild type (+/+), and 50% heterozygous mutant (+/-), and both female and male mice were used for the experiments. The genotype of each mouse was determined by PCR analysis of DNA isolated from tail tissue. Product sizes for the wild-type and disrupted nNOS genes are 117 base pairs and 280 base pairs, respectively.
The animals were kept under standardized conditions of temperature (21-22°C) and illumination (12 hours light - 12 hours darkness). They were allowed to adjust to this environment in cages with mesh bottoms for at least four days before the experiments began, with free access to tap water and pelleted food (Ewos, Södertälje, Sweden). The rats were deprived of food for 17-20 hours in groups of two to three before the experiments, but had free access to water right up to the beginning of the experiment.

All experiments were approved by the Uppsala University Ethical Committee for Animal Experiments.

Anesthesia and surgery

The rats were anesthetized with an i.p. injection of Inactin® (120 mg per kg body weight, Byk-Gulden, Konstanz, Germany). Spontaneous breathing was facilitated by a short PE-200 cannula placed in the trachea and the body temperature was maintained at 37.5±0.5°C by means of a heating pad controlled by a rectal thermistor. A PE-50 cannula containing heparin (12.5 IU ml⁻¹) dissolved in isotonic saline was placed in the right femoral artery to monitor blood pressure. For blood withdrawal, a PE 90 cannula, containing heparin dissolved in saline (100 IU ml⁻¹), was inserted into the left common carotid artery (studies I and IV). The right femoral vein was catheterized for administration of Ringer’s solution (to prevent dehydration, 120 mM NaCl, 2.5 mM KCl, 0.75 mM CaCl₂ and 25 mM NaHCO₃) either alone or with pentagastrin (studies I-III; 40 µg kg⁻¹h⁻¹) dissolved in the solution, at a rate of 1 ml per hour. The left femoral vein was cannulated when needed for drug infusion (study I).

The preparation of the gastric mucosa for intravital microscopy has been described previously (Holm-Rutili et al. 1985). A laparotomy was performed, the gastrohepatic ligaments were cut and the short gastric artery and vein were ligated and cut. After gentle exteriorization of the stomach through the midline abdominal incision, the forestomach was opened by electrical microcauterity. The rat was placed on its left side on a microscope stage with part of the corpus of the stomach loosely draped over a truncated cone at the center of the stage, with the mucosal surface facing upwards. A mucosal chamber, with a hole in the bottom corresponding to the position of the cone, was placed over the mucosa, exposing approximately 1.2 cm² of the surface, and the junction was sealed with silicone grease. The mucosal chamber was
filled with 5 ml of 0.9% NaCl kept at approximately 37°C by means of warm water circulating in a jacket in the bottom of the chamber.

Mouse anesthesia was induced by spontaneous inhalation of isoflurane (Forene®, Abbott Scandinavia AB, Kista, Sweden). The inhalation gas was administered continuously through a breathing mask (Simtec engineering) and contained a mixture of 40% oxygen, 60% nitrogen, and ≈2.2% isoflurane. Body temperature was maintained at 37°C by means of a heating pad controlled by a rectal thermistor probe. A catheter containing heparin (12.5 IU ml⁻¹) dissolved in isotonic saline was placed in the left carotid artery to monitor blood pressure. The jugular vein was cannulated for continuous infusion of Ringer’s solution at a rate of 0.35 ml h⁻¹. The gastric preparation is similar to that in rats, but the exposed area of the gastric mucosa is much smaller, 0.28 cm², and the chamber was filled with 2 or 3 ml saline.

Acid secretion

The solutions covering the gastric mucosa were changed at regular intervals of 10-15 min. Acid secretion was then measured by back-titrating the samples to the initial pH of the saline, using NaOH at concentrations of 1 or 10 mM. Acid secretion is presented as microequivalents of hydrogen ions secreted per minute and cm² of the exposed mucosa (µEq min⁻¹ cm⁻²). Pentagastrin (40 µg kg⁻¹ h⁻¹ i.v., studies I-III), the synthetic penta-peptide end-terminal of the hormone gastrin, or the H₂-receptor agonist imipramine (500µg kg⁻¹ h⁻¹ i.v., study I) was used to stimulate acid secretion. These doses each induce near maximal to maximal acid secretion in the rat. To inhibit acid secretion, either the H⁺/K⁺-ATPase inhibitor omeprazole (140 mg kg⁻¹ day⁻¹ or 3 x 70 mg kg⁻¹ day⁻¹ given by gavage, study II) or the H₂-receptor antagonist ranitidine (1 mg kg⁻¹ i.v. given as a bolus, study III) was used.

Juxtamucosal pH

In studies I and III the juxtamucosal pH (the hydrogen ion concentration in the mucus gel at the epithelial cell surface, pHₘ₅) was measured with hydrogen ion-selective microelectrodes. The microelectrodes were inserted into the mucus gel at an angle of 30-40° to the mucosa, by means of a
micromanipulator (Leitz, Wetzlar, Germany), and were placed just above the
surface epithelium under supervision through a stereomicroscope. Motility
and movements of the gastric mucosa made it necessary to check the
locations of the electrodes through the microscope during the experiments
and the electrodes were adjusted when needed. Glass tubing (borosilicate
tubing with omega dot, OD 1.2 mm, ID 0.9 mm; Frederik Haer & Co.,
Brunswick, ME) was pulled with a pipette puller (pp-83; Narishige Scientific
Instrument Laboratories, Tokyo, Japan) to a tip diameter of 1-3 µm. The
microelectrodes were siliconized at 200°C with tributylchlorosilane and
stored at 100°C. They were filled up to a distance of approximately 300 µm
from the tip with a proton cocktail (hydrogen ion Ionophore II-Cocktail).
The remaining part of the electrode was filled with HEPES buffer at pH 7.4,
connected by an Ag/AgCl wire to a dual differential electrometer with a high
input impedance (FD223; Biomedical Center, Uppsala, Sweden), and put in
a pipette holder (MEH3SF 1.2; Mark Finlay, WPI, Aston, England). The
reference electrode was filled with 3 M KCl, connected by an Ag/AgCl wire
to the ground of the electrometer, and placed in the saline covering the
gastric mucosa. To eliminate electrical disturbances, the experiments were
performed in a Faraday cage.

The electrodes were calibrated before and after the experiments in solutions
with a pH of 1.5-8 at 37°C. The solutions were made iso-osmolar (310
mOsm) with NaCl. The solutions with pH values of 1.5-3 were obtained by
adding 155 mM HCl to an unbuffered NaCl solution (155 mM), and those
with a pH of 4-8 by adding HCl or NaOH to a solution containing 10 mM
HEPES and 140 mM NaCl.

**Mucosal permeability**

Mucosal permeability (studies I and IV) was determined by measuring the
clearance of 51chromium-labeled EDTA (51Cr-EDTA) from blood to gastric
lumen (Nylander at al. 1989). The technique appears to provide a highly
reproducible measure of mucosal integrity and has the advantage that each
animal can serve as its own control (Bjarnason et al. 1985, Crissinger et al. 1990,
Hall et al. 1989). After completion of surgery and approximately 60 min
before the start of the experiment, 50-75 µCi 51Cr-EDTA was injected as a
bolus dose (0.5 ml), followed by a continuous intravenous infusion of 51Cr-
EDTA (10-30 µCi ml-1 in the Ringer solution) at a rate of 1.0 ml h-1. Four
0.2 ml blood samples were drawn during the experiment at intervals of
approximately 30 min. The first one was taken 60 min after the bolus
injection of $^{51}$Cr-EDTA. After each blood sample withdrawal, the blood volume loss was compensated for by injection of a 10% Ficoll 400 solution in saline. The blood sample was centrifuged and 50 µl of the plasma was removed for measurements of radioactivity (counts per minute, cpm). The gastric mucosa was covered with isotonic saline, which was replaced every 15 min. The luminal solution and the blood plasma were analyzed for $^{51}$Cr activity in a gamma counter (1282 Compugamma CS, Pharmacia, Uppsala, Sweden). In each experiment the various blood plasma $^{51}$Cr-EDTA activities were plotted against time and a straight line was drawn between the two nearest values. Each clearance value was calculated by dividing each individual effluent cpm value by a corresponding plasma cpm value. If there was less than 10% deviation between the different blood plasma cpms, a mean plasma cpm/ml value was calculated and used for all clearance samples. The part of the stomach that had been exposed in the chamber was cut out and weighed after the experiment. Clearance was calculated as:

\[
\text{Clearance} = \frac{\text{lumen sample (cpm ml}^{-1}) \times \text{sample volume (ml)} \times 100}{\text{plasma (cpm ml}^{-1}) \times \text{tissue weight (g)} \times \text{time (min)}}
\]

and is expressed as ml min$^{-1}$100 g$^{-1}$ wet tissue weight.

**Blood flow**

Laser Doppler flowmetry (LDF; Periflux Pf 2, Pf3 or Pf 4001, Perimed, Sweden) was used for blood flow measurements (studies I and IV). The nature of the Doppler shift from an illuminated tissue depends on the velocity and number of moving red blood cells (Ahn et al. 1985). The laser light (wavelength 633 nm, helium neon laser) is guided to the tissue by an optical fiber (standard probe, diameter = 0.7 mm) and the back-scattered light picked up by a pair of fibers of the same size. The penetration depth is dependent on the wavelength of the laser light, the distance between the fibers in the probe, and the properties of the tissue. Since the rat gastric wall is thin (1-2 mm), blood flow was probably measured through the entire wall of the illuminated portion. However, the recorded blood flow is mainly mucosal, since the amount of back-scattered light decreases exponentially with the depth of the tissue and about 80% of the total blood flow in the gastric wall is mucosal. Using this technique, blood flow is determined as a voltage output and expressed as percent of baseline values in 2.5-10 min periods. Blood flow was recorded continuously from the mucosal side of the gastric mucosa, with the probe fixed to a micromanipulator (Leitz, Wetzlar,
Germany) and kept at a distance of 0.5-1 mm above the surface of the mucosa, in the saline solution. The accuracy of the LDF technique for the gastrointestinal application has been evaluated and discussed earlier (Ahn et al. 1985, Holm-Rutili et al. 1986, Kvietsy et al. 1985).

Mucus gel thickness

Mucus gel thickness was measured (studies I and II) with micropipettes connected to a micromanipulator (Leitz) with a digimatic indicator (IDC Series 543, Mitutoyo Corp., Tokyo, Japan). The micropipettes were pulled with a pipette puller (pp-83; Narishige Scientific Instrument Laboratories, Tokyo, Japan) to a tip diameter of 1-3 µm, and siliconized to prevent mucus adhering to the glass, to allow repeated measurements at the same spot. The tip was dipped into a silicone solution (MS1107, 25% acetone) and dried at 100° C for 30 minutes. The luminal surface of the mucus gel was visualized by placing graphite particles (activated charcoal, extra pure, Merck, Germany) on the gel, and the epithelial cell surface was visible through the microscope. The micropipette was inserted into the mucus gel at an angle (a) of approximately 30° to the epithelial cell surface, and the distance (D) traveled by the micropipette from the luminal surface of the mucus gel to the epithelial cell surface was measured. The mucus thickness (T), which is the vertical distance between the cell surface and the luminal mucus surface, was then calculated from the formula T = D x sin a. The mean value of all measurements (4-5 different ones) on every measurement occasion was taken as one thickness value.

The loosely adherent mucus layer was removed by gentle suction under supervision through the stereomicroscope with a thin polyethylene cannula connected either to a syringe or to a weak vacuum pump. During this procedure, contact with the epithelium was carefully avoided.

Visualization of acidic channels in the mucus gel

A camera (Canon Ftb, Canon Inc., Tokyo, Japan, with film Kodak Ektachrome 320T) was connected to the stereomicroscope (Leica MZ12, Leica AG, Heerbrugg, Switzerland) and the gastric mucosa was transilluminated with light from a 150 W light source guided by fiberoptics. The pH-sensitive dye Congo red (blue below pH 3 and red above pH 5.2),
dissolved in saline to a concentration of 1 mM, was applied topically to stain the mucus gel. Thirty minutes after application, the Congo red was rinsed off and replaced by saline.

The adhering properties of Congo red to samples of the two different mucus layers were investigated by spectrophotometric measurements (Lambda 2, Perkin-Elmer GmbH, Überlingen, Germany) at 500 nm.

Real-time RT-PCR

RNA was isolated from scrapings of the mouse gastric mucosa and cDNA synthesis was performed with the Reverse Transcription System. The LightCycler FastStart DNA Master SYBR Green I was used for quantitative analyses of the generated cDNA. Calculations were performed as follows: The \( C_T \) represents the PCR cycle at which an increase in fluorescence above a baseline signal can be detected. The \( C_T \) value was used to calculate the amount of PCR product in comparison with the internal control, G6PDH. The \( C_T \) value for G6PDH was subtracted from the \( iNOS \) \( C_T \) value to obtain the mean \( \Delta C_T \) in each experimental group.

Administered drugs

The acid secretion modulating drugs pentagastrin, impromidine, ranitidine, and omeprazole have been described under Acid secretion in the Material and methods section. Indomethacin (3 mg kg\(^{-1}\), i.v.) inhibits prostaglandin synthesis through unselective inhibition of the cyclooxygenases COX-1 and COX-2. DIDS (4,4´-diisothiocyanostilbene-2,2´-disulfonic acid) was used to inhibit the apical Cl\(^-\)/HCO\(_3^+\) exchanger.

To inhibit NOS, the following agents were used: L-NNA (N\(^\omega\)-nitro-L-arginine), an unspecific L-Arg analogue; L-NIL (L-N\(^\phi\)-(1-iminoethyl)-lysine), an L-Arg analogue and iNOS-selective inhibitor; SMTC (S-methyl-L-thiocitrulline), an nNOS-selective slow-binding and L-Arg competitive inhibitor.
Statistical analysis

The results are expressed as means ± SE. For statistical evaluations of differences between data within a group, analysis of variance (ANOVA) for repeated measures was used, while ANOVA for multiple comparisons was performed when comparing data between groups. ANOVA was followed by Fisher’s protected least-significant difference test. Differences in pHjm within the same group and between groups of animals were evaluated statistically by analysis of variance in medians (Mann-Whitney test, studies I and III). To compare single values, Student's t-test for paired (study II) or unpaired data was used. All statistical calculations were performed on a Macintosh with the software Statview II™ SE Graphics (Abacus Concepts Inc., Berkeley, CA). The differences were regarded as significant if p<0.05.

Experimental protocols

The animals were allowed to rest for at least one hour after completion of the surgical procedures, and the experiments were not commenced until the mean arterial blood pressure, blood flow and acid secretion were stabilized.

Study I Intraluminal acid and gastric mucosal integrity: the importance of blood-borne bicarbonate.

The rats were divided into seven groups in respect to treatment, as shown below. Juxtamucosal pH (groups I-III) or blood flow and mucosal permeability (groups IV-VII) were measured before topical application of 100 mM HCl, in the presence of this acid, and after its removal.

<table>
<thead>
<tr>
<th>Juxtamucosal pH</th>
<th>Blood Flow and Mucosal Permeability</th>
</tr>
</thead>
<tbody>
<tr>
<td>I control</td>
<td>IV control</td>
</tr>
<tr>
<td>II pentagastrin</td>
<td>V impromidine</td>
</tr>
<tr>
<td>III NaHCO₃</td>
<td>VI pentagastrin</td>
</tr>
<tr>
<td></td>
<td>VII NaHCO₃</td>
</tr>
</tbody>
</table>
Pentagastrin (40 µg kg⁻¹ h⁻¹), imipramine (500 µg kg⁻¹ h⁻¹), and NaHCO₃ (5 mmol kg⁻¹ h⁻¹) were given as a continuous i.v. infusion throughout the experiments.

Study II Acid transport through channels in the mucous layer of rat stomach.

To examine the gastric mucus for acid channels, the pH sensitive dye Congo red (blue, pH <3; red, pH > 5.2; 1mM) was applied topically to 5 groups of rats receiving different treatments:

Group 1: Ringer’s solution i.v.
Group 2: pentagastrin 40 µg kg⁻¹ h⁻¹ i.v.
Group 3: omeprazole 400 µmol kg⁻¹ b.g. daily for seven consecutive days (140 mg kg⁻¹ day⁻¹)
Group 4: omeprazole 200 µmol kg⁻¹ b.g. every 8th hour for seven consecutive days (3 x 70 mg kg⁻¹ day⁻¹)

The channels were observed through a stereomicroscope and photographed with a camera connected to the microscope. The mucus thickness before and after removal of the loosely adherent layer was measured in groups 2-4, and the accumulation of the mucus was studied for 20 min.

Group 5: Ringer’s solution i.v.
Interactions between Congo red and the different mucus layers were investigated in group 5. Congo red was applied topically for 30 min followed by removal of the loosely adherent mucus layer. Congo red was applied for another 30 min, before the loosely adherent mucus was removed again and the firmly adherent mucus gently scraped off. The volume of mucus in the different samples could be calculated from the mucus thickness measurements made before each mucus removal. The absorbance of the different mucus layers was measured with a spectrophotometer at 500 nm.

Study III The importance of mucus layers and bicarbonate transport in preservation of gastric juxtamucosal pH.

The juxtamucosal pH was measured by hydrogen-sensitive microelectrodes placed in the mucus gel at the mucosal surface. Ten minutes after positioning
of the electrode, HCl (pH 1) was applied luminally in nine groups of rats receiving different treatments, as listed below. The mucus layers were left intact or the loosely adherent mucus layer was removed immediately before acid application.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Intact mucus</th>
<th>Mucus removed</th>
</tr>
</thead>
<tbody>
<tr>
<td>pentagastrin</td>
<td>I</td>
<td>II</td>
</tr>
<tr>
<td>pentagastrin and indomethacin</td>
<td>III</td>
<td>IV</td>
</tr>
<tr>
<td>ranitidine</td>
<td>V</td>
<td>VI</td>
</tr>
<tr>
<td>ranitidine and indomethacin</td>
<td>VII</td>
<td>VIII</td>
</tr>
<tr>
<td>pentagastrin and DIDS</td>
<td></td>
<td>IX</td>
</tr>
</tbody>
</table>

Pentagastrin (40 µg kg⁻¹ h⁻¹) was given as a continuous i.v. infusion throughout the experiments. Indomethacin (3 mg kg⁻¹, i.v.) was given as a bolus dose 60 min before luminal application of acid. Ranitidine (1 mg kg⁻¹, i.v.) was given as a bolus dose at least 30 min before luminal application of acid. DIDS (0.5 mM) was applied luminally for 15 min before the acid period. To facilitate diffusion of DIDS through the mucus, the loosely adherent mucus was removed before application of DIDS.

**Study IV Inducible Nitric Oxide Synthase is involved in acid induced gastric hyperemia in rats and mice.**

Gastric mucosal blood flow was measured in rats and mice before topical application of 155 mM HCl, in the presence of this acid, and after its removal. The rats were divided according to the specificity of the NOS inhibitor given and the mice used were either controls or had an inactivated gene coding for either the inducible or the neuronal isoform of NOS (see table below).

To investigate the damaging effect of 155 mM luminal acid, mucosal permeability was measured in a rat control group.

iNOS mRNA was detected by means of real-time RT-PCR in scrapings of mouse gastric mucosa. The gastric mucosa of group XI was scraped in unanesthetized mice immediately after spinal translocation. In groups XII-XIV the mice were anesthetized and operated on and the same protocol as
for groups V-IX (paper IV) was followed. Group XII was not exposed to luminal acid.

<table>
<thead>
<tr>
<th>Gastric mucosal blood flow</th>
<th>Permeability</th>
<th>RT-PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rats</td>
<td>Mice</td>
<td>Rats</td>
</tr>
<tr>
<td>I Control</td>
<td>V iNOS +/-</td>
<td>X Control</td>
</tr>
<tr>
<td>II L-NNA</td>
<td>VI iNOS +/-</td>
<td></td>
</tr>
<tr>
<td>III L-NIL</td>
<td>VII nNOS +/-</td>
<td></td>
</tr>
<tr>
<td>IV SMTC</td>
<td>VIII nNOS +/-</td>
<td></td>
</tr>
<tr>
<td></td>
<td>IX nNOS +/-</td>
<td></td>
</tr>
</tbody>
</table>

L-NNA, L-NIL and SMTC were all given in following dose: 10 mg kg$^{-1}$ i.v. bolus followed by 3 mg kg$^{-1}$h$^{-1}$ continuous i.v. infusion throughout the experiment.
Results and comments

Study I Intraluminal acid and gastric mucosal integrity: the importance of blood-borne bicarbonate.

For every hydrogen ion produced in the parietal cells and secreted into the gastric gland, one bicarbonate ion is transported over the basolateral membrane of the cell and into the microcirculation leading toward the surface of the mucosa. In this study we investigated the importance of this blood-borne bicarbonate originating from the parietal cells during acid secretion in maintaining a neutral pH$_{jm}$ during exposure of the luminal surface of the gastric mucosa to HCl at pH 1. The effect of luminal pH 1 on mucosal blood flow and permeability in acid- and non-acid-secreting mucosae was also studied.

**Juxtamucosal pH**

The juxtamucosal pH decreased from approximately 7.6 to 1.6 when HCl, pH 1, was applied topically to the gastric mucosa in rats not stimulated to produce acid (fig. 3). The pH gradient thus cannot withstand a luminal pH of 1 when endogenous acid secretion is not stimulated. However, when the luminal acid was changed to saline, the pH at the cell surface returned toward neutrality.

When acid secretion was stimulated with pentagastrin, luminal application of acid (pH 1) resulted in a small but significant decrease in pH$_{jm}$ to approximately 7 (fig. 3). This decrease was completely reversed by changing the luminal acid to saline.

Intravenous administration of NaHCO$_3$ prevented the acidification of the gastric mucosa as seen in the group not stimulated to produce acid. In the NaHCO$_3$ treated group, pH$_{jm}$ only decreased to 5 on topical application of HCl, pH 1, even though the animals were not stimulated to produce acid.
Again, pHjm returned immediately to a neutral pH when the luminal acid is changed to saline.

**Blood Flow and Mucosal Permeability**

When the acid secretion was not stimulated, topical application of HCl, pH 1, resulted in a 75% blood flow increase and a threefold increase in permeability. The blood flow was still significantly higher 20 min after the luminal acid had been changed to saline, compared to the baseline value. When acid secretion was stimulated with impromidine, the gastric mucosal blood flow was increased 35% by luminal pH 1, but the increase was reversed as soon as the luminal acid was changed to saline. When HCl at pH 1 was applied during pentagastrin-stimulated acid secretion, the gastric
mucosal blood flow increased by 25%, but this change was reversed by changing acid to saline. The mucosal permeability was not altered by luminal application of HCl, pH 1, in the groups where acid secretion was stimulated. In the group receiving NaHCO$_3$ intravenously, the gastric mucosal blood flow increased by 30% in the presence of luminal acid at pH 1, but the increase was reversed when the luminal solution was changed to saline. Mucosal permeability was not altered by luminal application of acid.

Conclusion: To maintain a neutral pH$_{in}$ in the presence of topical applied acid at pH 1, acid secretion had to be stimulated, since the blood-borne bicarbonate from the parietal cells was important in neutralizing back-diffused acid. Topical application of HCl at pH 1 increased the gastric mucosal blood flow. This increase was much larger when no stimulation of acid secretion occurred. Mucosal permeability was increased by luminal pH 1 only when acid secretion was not stimulated.

Study II Acid transport through channels in the mucous layer of rat stomach.

Since a pH gradient exists in the mucus layer covering the gastric mucosa even during endogenous acid secretion, the secreted acid must traverse the mucus gel only at restricted sites. The aim of this study was to visualize these paths in which acid passed toward the gastric lumen.

When the mucus gel had been stained with Congo red (blue, pH<3; red, pH>5.2), both red and blue colored crypt openings were detected in the group with spontaneous acid secretion. Thread-like structures leading through the mucus toward the lumen were attached to the crypt openings. When acid secretion was stimulated with pentagastrin, the Congo red dye colored the entire surface of the mucus layer blue. The loosely adherent mucus layer was then removed and the pH-sensitive dye had not reached the mucosal surface but seemed to have precipitated within its acidic range. Congo red was therefore applied once again, and blue-colored crypt openings with accompanying blue channels (paths) appeared all over the mucosa. Omeprazole given once a day for one week acutely inhibited the acid secretion when the experiments were commenced, and red-stained crypt openings with attached channels were identified. Eight to nine hours after the last dose of omeprazole, acid secretion began, and the crypt openings and channels turned blue (fig. 4). When omeprazole was given every eighth hour for one week, acid secretion was completely inhibited and the mucus layer
was probably renewed. In this group, no red-stained channels could be identified, not even when luminal acid was instilled and the red-stained crypt openings turned blue. These results indicate that the channels are not formed with the secretion of the firmly adherent mucus layer, but are created during acid secretion.

The mucus thickness was measured in the pentagastrin- and omeprazole-treated groups. The total mucus layer was significantly thicker in the group given omeprazole once daily. When the loosely adherent mucus layer had been removed, no mucus accumulation occurred in the omeprazole-treated groups, in contrast to the pentagastrin-treated group. This could imply that the paths are important not only for acid transport but also for the mucus produced by mucin-producing cells inside the gastric gland, i.e., the mucus neck cells, and that the loosely adherent mucus consists mainly of mucus originating from these cells.

Figure 4. Blue colored crypt openings with attached channels in the mucus covering an acid secreting gastric mucosa seen from above.
To further elucidate the question whether the mucus of the different mucus layers was of different types, the interaction with Congo red was studied with a spectrophotometer. Congo red attached to a significantly smaller extent to the firmly adherent than to the loosely adherent layer. This could indicate that the mucins making up the different mucus layers are differently glycosylated.

In summary, in this study, for the first time, channels or paths in the mucus gel, leading from the crypt openings towards the gastric lumen, were visualized. Transport of acid secretion, and perhaps also of mucus from the mucous neck cells, was restricted to these channels.

Study III The importance of mucus layers and bicarbonate transport in preservation of gastric juxtamucosal pH.

In this study, we investigated the importance of the loosely adherent mucus layer for the maintenance of the juxtamucosal pH (pHjm) in the presence of luminal pH 1 during different acid secretory states (stimulated or inhibited). The influence of prostaglandin-stimulated bicarbonate secretion on pHjm was also studied. To investigate the route by which bicarbonate passes through the cells, the apical Cl-/HCO3- exchanger was inhibited with DIDS during stimulated acid secretion.

Removal of the loosely adherent mucus layer did not influence pHjm during stimulation or inhibition of the acid secretion and in the presence of luminal pH 1. Inhibition of prostaglandin synthesis did not further decrease pHjm when acid secretion was stimulated either if the mucus was left intact or if the loosely adherent mucus layer had been removed. When both acid secretion and prostaglandin synthesis were inhibited, removal of the loosely adherent mucus layer caused a further but non-significant decrease in pHjm in the presence of luminal pH 1, and led to a significantly slower re-establishment of the pH gradient after the acidic period. This indicates that the loosely adherent mucus layer is important for pHjm only in a situation where both acid secretion and prostaglandin synthesis are inhibited.

When pentagastrin-treated rats were pretreated with DIDS and acid at pH 1 was topically applied, pHjm decreased to a lowest level of 1.4 (fig. 5).
The finding that pH jm in the pentagastrin-stimulated groups was still neutral in the presence of luminal HCl at pH 1, indicates that bicarbonate traverses the surface epithelium through a DIDS-sensitive mechanism, presumably the apical Cl-/HCO₃⁻ exchanger.

In conclusion, the loosely adherent mucus layer does not seem to be important in preserving pH jm in the presence of luminal pH 1. DIDS-sensitive bicarbonate transport from the alkaline blood during ongoing acid secretion is essential for maintenance of a neutral pH jm, whereas prostaglandin-stimulated bicarbonate secretion contributes only to a minor extent and only when acid secretion is inhibited and the loosely adherent mucus layer is removed.
Study IV Inducible nitric oxide synthase is involved in acid induced gastric hyperemia in rats and mice.

When acid together with a barrier-breaker is applied to the gastric mucosa, gastric mucosal blood flow increases and hemorrhagic lesions are formed. This increase in mucosal blood flow has been shown to occur via a release of NO. As reported in paper I, it was found that gastric mucosal blood flow increased on application of luminal acid alone. We hypothesized that NO was also important in this hyperemia, and the NOS isoform involved was investigated in rats treated with different specific NOS inhibitors and in mice in which the gene coding for iNOS or nNOS had been inactivated.

When HCl (155 mM) was applied topically to the rat or mouse gastric mucosa, the gastric mucosal blood flow increased significantly. When rats were pretreated with the unspecific NOS inhibitor L-NNA, the iNOS-specific inhibitor L-NIL or the purported nNOS-specific inhibitor SMTC, this hyperemia was blocked. SMTC transiently increased the mean arterial blood pressure, indicating that it also had an inhibitory effect on eNOS. Knock-out mouse models with certain genes inactivated is another tool for investigating the role of specific enzymes, and in this study iNOS -/- (fig.6) but not nNOS -/- showed an attenuated response to luminal acid.

![Image](image.png)

Figure 6. Gastric mucosal blood flow (LDF%) in control mice (+/+, n=6) and iNOS knockout mice (-/-, n=6) presented as percent of control period, time 15-20 min. HCl (155mM) was applied luminally. Values are expressed as mean ± SE. * p<0.05 compared with the control period before acid application.
Inducible NOS mRNA had not previously been detected under unpathological conditions in the gastric mucosa. However, with real-time RT-PCR we found iNOS RNA in scrapings of mouse gastric mucosa. The amount of RNA was not upregulated either by anesthesia, the gastric preparation, or luminal acid.

It is concluded that iNOS is involved in the acid-induced gastric hyperemia and seems to be constitutively expressed in the gastric mucosa.
Discussion

The gastric mucosa is continuously exposed to high acidities and requires efficient defense mechanisms working in unison for its preservation. The ambition with the present studies was to increase the understanding of how the gastric mucosa is able to resist the strong luminal acid to which it is exposed on a regular basis. More specifically, pre- (juxtamucosal pH, mucus layer, acid and bicarbonate transport), and postepithelial defense mechanisms (mucosal blood flow), as well as the permeability of the gastric mucosa, were investigated.

What is the pH at the epithelial cell surface during acid secretion and exposure to strong luminal acid?

A mucus layer wherein a pH gradient can be formed covers the entire gastric mucosa. The pH gradient protects the gastric mucosa against back-diffusing acid from the lumen. Earlier studies of the pH gradient and the pH at the gastric epithelial cell surface (pH$_{jm}$) were generally conducted in a non-acid-secreting situation, and a total collapse of the gradient was observed when the luminal pH decreased below 1.4 (Patronella et al. 1988, Ross et al. 1981). However, luminal acidity is a result of endogenous acid secretion, and investigation of the pH gradient during stimulation of acid secretion therefore seems highly relevant. In addition, acid secretion has been found to have a protective effect on mucosal resistance against luminal acid, since a smaller fall in potential difference was found in acid-secreting than in non-acid-secreting mucosae following topical application of acid (O’Brien et al. 1976). Results from our laboratory showed the existence of a pH gradient during acid secretion in the presence of luminal acid at pH 2 (Schade et al. 1994).

Acid secretion is stimulated by the smell of food or even by thinking of food (the cephalic phase, vagus activation). During the cephalic phase, the stomach might be empty, with no luminal contents to buffer the produced
acid, resulting in a dramatic drop of luminal pH. In addition, in between meals, the gastric lumen pH can be very low, even though acid secretion is not stimulated (Teyssen et al. 1995). We mimicked these conditions and applied acid with a pH of 1 luminally in non-acid-producing rats and in rats in which acid secretion was inhibited or stimulated (studies I and III). We found that in the non-acid-producing rats, with low acid secretion, pH$_{jm}$ decreased significantly to approximately 1.6±0.2 during application of luminal pH 1. When acid secretion was inhibited with ranitidine and HCl at pH 1 was applied topically, a similar drop of pH$_{jm}$ to 2.2±0.5 was observed. In both situations, pH$_{jm}$ returned to neutrality when the acid was changed to saline, which is not consistent with initiation of a sustained injury of the mucosa. However, during pentagastrin-stimulated acid secretion the pH of the gastric epithelial cell surface was neutral even when HCl in a concentration of 100 mM was applied topically. It seems reasonable to conclude that ongoing acid secretion is a prerequisite for preservation of a functional intra-mucus pH gradient in the presence of luminal pH 1 and that a blood-borne alkaline tide provides the gastric epithelium with the HCO$_3^-$ needed for preservation of a neutral pH$_{jm}$.

**Bicarbonate transport**

Bicarbonate needs to be persistently secreted from the surface epithelial cells to meet and neutralize back-diffused acid. Gastric bicarbonate secretion is difficult to measure during acid secretion, since the set-up has to be CO$_2$ impermeable. Because of this inconvenience, bicarbonate secretion in the stomach has most often been measured during inhibition of acid secretion. In the studies presented here bicarbonate secretion was estimated indirectly by measuring the pH$_{jm}$ at the epithelial cell surface in both acid-secreting and non-secreting mucosae.

**The alkaline tide**

When acid secretion is stimulated, the blood passing the parietal cells on its way to the surface epithelium is alkalinized by bicarbonate. The arrangement of the capillaries along the gastric glands is optimal for transporting this alkaline tide. As discussed earlier, we found that on-going acid secretion is required for preservation of a neutral pH$_{jm}$ in the presence of luminal pH 1, indicating that the blood-borne bicarbonate is important for neutralization of back-diffused acid in the mucus. In accordance with this finding, Kivilaakso
(1981) reported that i.v. infusion of NaHCO₃ causing high-HCO₃⁻ metabolic alkalosis significantly decreased the incidence of acid-induced mucosal injury. Respiratory alkalosis of similar degree had no protective effect against luminal acid, indicating that it is HCO₃⁻ and not the alkalinity per se that is important.

When HCO₃⁻ was infused intravenously in rats with no ongoing acid secretion (study I), pH jm was only slightly reduced by topical application of acid at pH 1. The above results confirm that blood-delivered bicarbonate is involved in preserving the pH gradient during acid secretion. However, the pH gradient was not as efficient as in the acid-secreting groups, probably because the HCO₃⁻ concentration was not as high as it is locally in the mucosal capillaries during endogenous acid secretion. In dogs, the concentration of arterial bicarbonate increased by 5 mM after feeding (Ozaki et al. 2000), and the local concentration of bicarbonate in the gastric mucosa must be much higher. However, the basal pH jm before application of luminal acid was independent of the acid secretory status (studies I and III), indicating that the alkaline tide per se did not stimulate a basal bicarbonate secretion.

When acid secretion was stimulated in the present studies, topical application of acid at pH 1 generally caused a transient decrease in pH jm from approximately 7.4 to 6 or just below during the first minutes before it returned to a neutral level. Thus it seems that the bicarbonate secretion is increased by luminal acid, and that it takes a few minutes for it to become observable. An unknown factor is what triggers the increase in bicarbonate secretion when acid is applied luminally. The transient drop from pH 7.4 to 6 might be the triggering signal, but how this signal is transmitted or what it activates is not yet known. There might have been a rapid decrease in pH jm to an even lower level than that recorded, which in that case we would have missed on account of the slowness of the hydrogen-sensitive microelectrodes. One result of the reaction between H⁺ and HCO₃⁻ is an increase in CO₂ tension, and it has been suggested that it is CO₂ and not the hydrogen ion per se that signals for increased bicarbonate secretion in response to luminal acid in the rat duodenum (Holm et al 1998). When rat duodenum was perfused with a near neutral solution with a high CO₂ tension, the bicarbonate secretion was stimulated. The non-specific NOS inhibitor L-NAME, but not inhibition of prostaglandin synthesis, blocked this increase in bicarbonate secretion. If it is CO₂ and not H⁺ that is the triggering signal, this could explain how mucosal bicarbonate secretion is increased by luminal acid even though the pH gradient is not greatly affected, and how this secretion is regulated also in the stomach.
Interestingly, when luminal acid was changed to saline in our studies, pH\textsubscript{jm} returned immediately to a neutral level, and an overshoot, e.g. alkalization of the epithelium, was never observed. Thus it seems that the regulation of bicarbonate secretion is a rapid and a very sensitive process.

**Prostaglandin-stimulated bicarbonate secretion**

How important is the prostaglandin-dependent bicarbonate secretion for the maintenance of pH\textsubscript{jm}? In rats not stimulated to produce acid, inhibition of the prostaglandin synthesis with aspirin in the presence of luminal acid at pH 2 resulted in a decrease in pH\textsubscript{jm} compared to that in a control group (Ross et al. 1983). In accordance with these findings, when acid secretion and prostaglandin synthesis were both inhibited, we found that the pH at the cell surface was slightly reduced in the control situation before acid was applied in the lumen (study III). Interestingly, after topical application of HCl (155 mM), inhibition of prostaglandin synthesis did not further reduce pH\textsubscript{jm}, whether acid secretion was stimulated or inhibited. It is possible that the strong acid that penetrates down to the surface epithelial cells when acid secretion is inhibited conceals a small fraction of bicarbonate secretion that is prostaglandin-dependent. These results indicate that gastric prostaglandin-dependent bicarbonate secretion is involved in basal bicarbonate secretion when no acid secretion occurs, but does not seem to be important in neutralizing back-diffused acid during acid secretion.

**DIDS-sensitive bicarbonate transport**

Our results convincingly show that the pH gradient is better preserved in an acid-secreting stomach than in a resting one. Thus, during endogenous acid secretion the gastric microcirculation supplies the surface epithelial cells with the bicarbonate needed for juxtamucosal neutralization. When DIDS had been applied luminally and acid secretion stimulated with pentagastrin, pH\textsubscript{jm} decreased dramatically when HCl at pH 1 was applied topically in the lumen (study III). This indicates that bicarbonate is transported through a DIDS-sensitive mechanism. Experiments in vitro have shown that gastric bicarbonate secretion is dependent on luminal chloride ions, indicating the presence of an apical Cl\textsuperscript{-}/HCO\textsubscript{3}\textsuperscript{-} exchanger (Flemström 1980). Bicarbonate has been reported to enter the surface epithelial cells basolaterally in cotransport with sodium (Curci et al. 1994, Rossman et al. 1999). DIDS is an inhibitor of the Cl\textsuperscript{-}/HCO\textsubscript{3}\textsuperscript{-} exchanger as well as of Na\textsuperscript{+}/HCO\textsubscript{3}\textsuperscript{-} cotransport. In the parietal cell, a basolateral Cl\textsuperscript{-}/HCO\textsubscript{3}\textsuperscript{-} exchanger has been found and inhibition of this
transporter inhibited acid secretion (Horie et al. 1992). In the concentration used in this study, DIDS applied luminally to the gastric mucosa seemed to inhibit at least the apical Cl⁻/HCO₃⁻ transporter in the surface epithelial cells. Since the acid secretion was not reduced after DIDS treatment, no major amount of the inhibitor appeared to have entered the circulation.

However, it is also possible that DIDS might influence the paracellular transport of HCO₃⁻, which normally is very restricted (Allen et al. 1993).

How important are the mucus layers?

As early as in 1856 Dr Bernard wrote that the mucus coating the stomach rendered it impermeable to the acidic gastric juice, likening it to a porcelain vase. Today we know that the mucus layer covers the gastric mucosa as a continuous coat (Allen 1989), and it is believed that it contributes to the preepithelial defense mechanisms in different ways. In the Necturus antrum a luminal mucus layer has been shown to be necessary for keeping the juxtamucosal and intracellular pH neutral in the presence of luminal acid (Kiviluoto et al. 1993). In accordance with this finding, an unstirred layer has been demonstrated in which the pH gradient is formed by secreted bicarbonate, neutralizing back-diffused acid (Ross et al. 1981, Schade et al. 1994). It has been difficult histologically to study the mucus, since it easily becomes dehydrated and eroded during the preparation. However, two cell types secreting different mucins have been identified in the corpus part of the stomach, namely surface epithelial mucous cells (MUC5AC) and mucous neck cells (MUC6) (Ota et al. 1991, VanKlinken et al. 1997). We have previously observed two separate mucus layers covering the gastric mucosa, a loosely adherent layer that is easily removed by suction, and a layer that firmly adheres to the gastric epithelium (Atuma et al. 2001). The latter layer cannot be removed by suction or rubbed off with a cotton tip. The thicknesses of the different layers can be measured with micropipettes inserted into the mucus. With this method the total mucus thickness (loosely adherent plus firmly adherent) was found to be 189±11 µm and the thickness of the firmly adherent layer (measured directly after removal of the loosely adherent layer) was 80±5 µm (Atuma et al. 2001). An increase in thickness is a normal defensive response to luminal insult, and it is generally believed that the thicker the mucus, the better the protection (Allen 1989).

In study III we investigated how removal of the loosely adherent mucus layer influenced pHᵢₑₙ in the presence of HCl, pH 1, in the lumen. The results

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clearly show that the loosely adherent layer is of minor importance in maintaining the pH<sub>jm</sub>, since removal of this mucus layer caused no further changes of pH<sub>jm</sub>, compared to the control group. This seems plausible, since the loosely adherent layer most probably is removed from the mucosal surface by ingested food, which also stimulates acid secretion. One function of the loosely adherent layer might be to lubricate food particles and bind bacteria. In earlier experiments in which the pH gradient was measured through the entire mucus layer (firmly and loosely adherent), the depth of this gradient in the mucus during acid secretion (~100 µm closest to the epithelial surface, luminal pH 2 or 3) was found to correspond quite well with the thickness of the firmly adherent mucus layer (Atuma et al. 2001, Johansson et al. 1998, Schade et al 1994). This supports the finding from the present study that the loosely adherent layer is not important for preserving a pH gradient. The only situation where we found that the loosely adherent layer influenced pH<sub>jm</sub> was when both acid secretion and prostaglandin synthesis were inhibited. Under these conditions removal of the loosely adherent mucus layer resulted in a slower recovery to a neutral pH after application of luminal acid. This could indicate that in a non-acid secretion situation, prostaglandin-stimulated bicarbonate secretion contributes to neutralization of back-diffused acid. However, we have found that the thickness of the inner firmly adherent mucus layer is increased after topical treatment with PGE2, and correspondingly decreased after pretreatment with indomethacin (Johansson et al. 1998). Hence, the remainder of the firmly adherent mucus layer was perhaps too thin to establish an efficient neutralization zone for acid and bicarbonate. Further studies are required to elucidate the importance of the thickness of the firmly adherent mucus layer in maintaining the pH<sub>jm</sub>.

On the basis of these results, the simplified assumption that the thicker the mucus layer, the better the protection needs to be revised and efforts should be directed toward understanding the regulation and contents of the firmly adherent mucus layer.

How is acid transported through gastric mucus?

Acid is secreted into the lumen of the gastric glands and has to pass through the mucus layer to reach the gastric lumen. Since we invariably found a neutral or slightly alkaline pH at the epithelial cell surface during acid secretion, the acid must have penetrated the mucus layer from the site of
production to the lumen of the stomach without acidifying the epithelial cell surface. Thus, unrestricted diffusion as a major acid transport mechanism can be ruled out.

In a previous study, acidic spots in the mucus gel during acid secretion were observed after staining the mucus with the pH-sensitive dye Congo red (blue, pH<3; red, pH>5.2, Holm et al. 1990). We have now succeeded in identifying channels for transport of acid through the mucus layer covering the gastric mucosa after staining the mucus with Congo red (study II). This treatment revealed blue-colored crypt openings with attached thread-like channels in the mucus during acid secretion. These channels are most probably created by high intraglandular pressure (Holm et al. 1992), pushing acid and mucus from the gland lumen into the firmly adherent mucus layer and leaving a path with a structure different from that of the surrounding gel. The existence of channels transporting glandular secretions to the gastric lumen would also explain how the large molecules pepsinogen/pepsin and intrinsic factor traverse the mucus layer, since diffusion is restricted for molecules of their size (Allen 1989, Allen et al. 1993).

A channel occasionally became attached to the micropipette and could be pulled out of the mucus layer, indicating that the wall of the channel has a firm configuration. After the mucus had been stained with Congo red, the channels, with an outer diameter of 5-7 µm, could be seen to be pushed in front of the microelectrode or moved sideways. In accordance with these observations, we have in all our measurements of pH gradients and pHjm only once been able to penetrate a channel with a microelectrode and record a low pH during acid secretion (Schade et al. 1994, studies I and III). The structure of the channel wall is not known. However, in the corpus part of the stomach two cell types secreting different mucins have been identified, surface mucous cells and mucous neck cells (MUC5AC and MUC6, respectively (Ota et al. 1991, VanKlinken et al. 1997). The two types of mucins differ considerably in their histochemical properties (Ota et al. 1991). In absorbance experiments on samples of the different mucus layers, we found that Congo red interacted significantly more strongly with the loosely adherent layer than with the firmly adherent one. In addition, Congo red was concentrated at the channel structures or adhered to the channels, irrespective of the state of acid secretion. Thus we hypothesize that the loosely adherent mucus layer and the channel wall consist mainly of the same mucins and that mucus from mucous neck cells is pushed out from the crypts through the channels, forming the loosely adherent layer. In addition, the channel in itself might consist of mucus from the mucous neck cells.
Until proper antibodies directed toward the protein core of the rat mucins become available, this question remains speculative.

Studies of acid transport through the mucus have been conducted by many researchers for several decades, resulted in various explanatory models. Chu et al. (1999) suggested that acid diffuses from its site of secretion toward the lumen, since with an inverted confocal microscope and pH sensitive dyes they found a reversed pH gradient with pH 5 in the lumen and pH 3.5 at the cell surface during pentagastrin stimulation. The reason for absence of channels in their study might have been that the intraglandular pressures in their preparations were not sufficiently high to create them.

Schreiber and Scheid (1997) suggested a model for transport of protons from the gland to the lumen, where acid was bound to and buffered by the mucus in the glands and was then transported together with the continuously formed mucus toward the lumen. In their model, pepsinogen was also transported within the mucus layer, and when converted into pepsin could degrade the mucus and release the acid. Their model is based upon the acid secretion rate in vitro, which is much lower than the acid secretion measured in vivo (Batzi et al. 1986). In addition, in their model mucus is constantly secreted at a very high rate, something which has not been described in vivo. In an in vivo situation, an unrealistically high mucus secretion would be required for buffering and binding secreted acid.

When acid is injected under pressure into mucus in vitro (Bhaskar et al. 1992), a phenomenon called viscous fingering occurs provided the mucus has a pH above 4. This is a process in which a fluid of lower viscosity, injected into one of higher viscosity, penetrates rather than displaces the stationary solution. Translated into our model, where we have found a high pressure in the gastric glands (Holm et al. 1992) and a neutral pH in the mucus at the epithelial cell surface (Schade et al. 1994, studies I and III), the channels we have observed in vivo could represent the viscous fingers demonstrated earlier in vitro. Bhaskar et al. (1991) have also found an increase in mucus viscosity as the pH was lowered, which was reversed when the pH increased. However, our channels seemed to be independent of pH, as they were also observed when no acid secretion occurred.
Influence of acid on gastric mucosal blood flow and mucosal permeability

We found that when acid secretion was not stimulated, topical application of HCl, pH 1, resulted in a 75% blood flow increase and a threefold increase in mucosal permeability (study I). Compared to the baseline value, the blood flow was still significantly higher 20 minutes after the luminal acid had been changed to saline. When NaHCO3 was given i.v. or acid secretion was stimulated with pentagastrin or impropidine, the gastric mucosal blood flow was only increased 25-35% by topical application of HCl, pH 1, and the increase was reversed as soon as the luminal acid was changed to saline. The mucosal permeability was not altered by topical application of HCl, pH 1, in these groups. Again, blood-borne bicarbonate seemed to be vital in the mucosal protection against luminal acid. Combined with the results of the pHjm measurements, these findings indicate that when the concentration of the luminal acid overcomes the bicarbonate secretion needed for neutralization of the acid in the mucus gel, the epithelial surface becomes acidified and hydrogen ions can diffuse into the mucosa as the gastric permeability increases. The increase in mucosal blood flow was significantly greater when low pHjm and increased permeability were detected.

Earlier studies have shown that, in combination with a barrier-breaking substance (e.g., ethanol or sodium taurocholate), acid increases the gastric mucosal blood flow and causes hemorrhagic lesions when applied luminaly to the gastric mucosa (Barreto et al. 1993, Li et al. 1992). The mechanism underlying this acid-induced gastric mucosal hyperemia involves activation of sensory afferent nerves, leading to release of CGRP in the vicinity of the submucosal arterioles (Li et al. 1992). CGRP acts on the vascular endothelium lining these vessels, resulting in generation of NO (Holzer 1998). NO diffuses to the underlying vascular smooth muscle cells, where it stimulates soluble guanylate cyclase, leading to elevated cGMP levels and relaxation of the vascular smooth muscle. This increase in gastric blood flow can be blocked with a non-selective NOS inhibitor (Lippe et al. 1992). In addition, impairment of the neurally mediated hyperemic response through disruption of the sensory afferent nerves, antagonism of CGRP receptors, or blockade of NO synthesis, results in a significant increase in the susceptibility of the gastric mucosa to damage (Holm et al. 2001, Li et al. 1992, Lippe et al. 1992). We found that the gastric mucosal blood flow was increased by luminal acid alone, without the addition of a barrier-breaker. This hyperemia was greater when no acid secretion occurred. Whether the mechanism underlying this acid-induced hyperemia also involves CGRP-releasing nerves is not known.
How these nerves are activated to release CGRP when no mucosal acidification takes place is also unknown.

The role of inducible NOS in the gastric hyperemia in response to luminal acid

We found that iNOS is involved in the gastric hyperemia occurring in response to luminal acid and that iNOS has a protective role in the gastric defense in this context (study IV). Our results demonstrated that application of luminal acid (HCl, 155 mM) on the gastric mucosa caused a hyperemia that was blocked in iNOS -/- mice and by selective inhibition of iNOS (L-NIL treated rats). Earlier studies have failed to reveal the presence of iNOS in the gastric mucosa (Konturek et al. 1998, Price et al. 1996). However, using real-time RT-PCR in mouse gastric mucosa we found iNOS mRNA expression at a level not influenced by anesthesia, preparation of the gastric mucosa, or luminal acid. These results indicate the possibility of posttranscriptional regulation of iNOS activity that differs from the regulation occurring in the macrophages. Interestingly, iNOS has been found to exist in a constitutive way (or to be constantly induced) in other organ systems that are exposed to the exterior, and therefore must function as an effective barrier inhibiting the entrance of unwanted agents into the system. In the epithelial cells of the respiratory tract (in airways and paranasal sinuses) (Guo et al. 1995, Lundberg et al. 1995), the high levels of tonic expression of iNOS and NO production have special relevance for airway defense mechanisms (Jain et al. 1993, Schmidt et al. 1994). iNOS is also expressed in a seemingly constitutive way under normal conditions in the esophageal epithelium (Casselbrant et al. 2002), in parts of the small intestine (duodenum and ileum) (Hoffman et al. 1997, Holm et al. 2001), and in occasional patches in the colon (McCafferty et al. 1999), and is possibly involved in the mucosal defense. The constant presence of iNOS in these organ systems could reflect the regular challenge by bacteria, viruses and fungi, and so on.

In summary, the resistance of the gastric mucosa to strong acid is dependent on a network of defense mechanisms cooperating at different levels. A neutral pH_{jm} is dependent on the alkaline tide supplying a sufficient amount of bicarbonate and probably also by a firmly adherent mucus layer. Acid secretion is transported in restricted channels through the mucus, enabling pH_{jm} to remain neutral even during ongoing acid secretion. Luminal acid increases gastric mucosal blood flow through iNOS activation.
Conclusions

The studies presented here have uncovered several different ways in which the gastric mucosa resists its intrinsic acid and have provided further insight into the fascinating mechanisms of gastric mucosal protection.

More specifically the following were found:

The pH gradient in the mucus gel restrains back-diffusion of luminal acid. The existence of the pH gradient is made possible by bicarbonate transported by the blood flow from the acid secreting parietal cells to the surface epithelium, where a DIDS-sensitive bicarbonate transport through the epithelium occurs.

Prostaglandin-dependent bicarbonate secretion seems to be less important in maintaining a neutral pH\textsubscript{jm}.

Of the mucus gel layers, only the firmly adherent one is important for the pH\textsubscript{jm}. Accordingly, the pH gradient is found in the 80\textmu m thick mucus gel layer closest to the mucosa.

Secreted acid penetrates the mucus gel through specific channels only. Thus, a pH gradient with a neutral pH\textsubscript{jm} can still exist during acid secretion.

Gastric mucosal blood flow increases on application of strong luminal acid. This acid-induced hyperemia involves the inducible but not the neural isoform of NOS.
Future perspectives

These studies have shown that an acid-secreting mucosa can resist topical 100 mM HCl by virtue of the existence of a pH gradient in the mucus layer. From pilot experiments we know that a pH gradient also occurs in the antral part of the stomach in the presence of topical acid, pH 1. Parietal cells are not found in the antrum and hence no acid secretion with a subsequent alkaline tide provides the mucosa with bicarbonate. The way in which the antrum resists strong luminal acid thus remains to be elucidated.

It would be interesting to study the involvement of the basolateral NHE2 found on gastric surface epithelial cells in bicarbonate transport during acid secretion by measuring pHjm in NHE2-/- mice.

I believe that failure to create channels could cause gastric damage. The features of the channels for acid transport in the mucus are unknown and need to be clarified. Also, the glandular pressure is most likely crucial for the formation of channels. If the pressure is too low, channels will probably not be formed, leading to acidification of the epithelium with consequent damage.

A logical next step of great interest would be to further characterize the different mucus layers covering the gastric mucosa, in an attempt to understand the different viscosities and physiological importance of these layers. Firstly, it is necessary to determine the mucin contents in the different mucus gel layers.

The influence of the firmly adherent mucus layer in the preservation of pHjm is still unclear. It seems plausible to conclude, however, that an unstirred layer of mucus, is a prerequisite for formation of a pH gradient. To study factors and situations that reduce the mucus thickness beneath its critical value below which a pH gradient cannot be maintained, would be of huge interest.
In mice, we have found iNOS mRNA in a constant amount in the gastric mucosa independently of anesthesia, operation, and luminal acid. The exact location of iNOS in the gastric mucosa is not yet known, however. We have tried using immunohistochemistry to stain iNOS with antibodies directed toward the mouse macrophage iNOS, but this treatment did not stain anything in the mouse mucosa. We hypothesize that the constitutively expressed iNOS is somewhat different to the iNOS expressed by the immune cells, and that other antibodies need to be tried.

It would also be interesting to study the involvement of the mucosal iNOS in other mucosal defense mechanisms, such as mucus secretion and mucosal permeability.
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References


Svensk sammanfattning av de i avhandlingen inkluderade artiklarna

I. Intraluminal acid and gastric mucosal integrity: the importance of blood-borne bicarbonate.

Synderstad I, Johansson M, Nylander O & Holm L.
Am J Physiol Gastrointest Liver Physiol 2001;280:G121-G129.


Magsäcken i Inactin®-sövda hanråttor frilades och korpusdelen monterades med slemhinnan uppåt för intravitalmikroskopi och täcktes av varm fysiologisk koksaltslösning, pH-känsliga mikroelektroder, förbundna med en mikromanipulator, fördes in i slemsmet med en vinkel på ca 30° mot slemhinnan. Mikroelektroden placerades precis ovanför ytepitelcellerna under uppsikt genom ett stereomikroskop. I andra försök måttes blodflödet med laser–Doppler flödesmetri (LDF), som ger ett mått på procentuell förändring i blodflödet jämfört med en kontrollnivå. Permeabiliteten uppskattades med hjälp av clearance av 51Cr-EDTA, som getts intravenöst,
från blod till maglumen. Efter en stabil kontrollperiod lades HCl pH 1 på magslekhinnan i två tiominuters perioder. Syran sköljdes noggrant bort och försöket fortsatte i ytterligare trettio minuter. Syrasekretionen stimulerades med pentagstrin eller impromidin.

**Juxtamukosalt pH**

I djur där syrasekretionen ej stimulerades, orsakade pH 1 i lumen en kraftig sänkning av pH jm från pH 7.6 till pH 1.6. Detta ska jämföras med när syrasekretionen stimulerades då pH jm sjönk till enbart pH 7. I djur som ej producerade syra men fick HCO₃⁻ infusion under försöket, hölls pH jm betydligt mer neutalt och sjönk till enbart pH 5 under luminal syra. Detta påvisar att bikarbonatkoncentrationen i blodet är viktigt för upprätthållandet av neutrat pH vid ytepitelet vid syra i lumen.

**Slemhinnans blodflöde och permeabilitet**

När syrasekretionen ej stimulerades, gav lumen pH 1 upphov till en blodflödesökning på 75% och en tredubblad permeabilitetsökning. Då syrasekretionen stimulerades eller bikarbonat gavs intravenöst ökade blodflödet enbart 25%-35% av pH 1 i lumen, och ingenökning av permeabiliteten sågs.

Sammantaget visar dessa resultat att den extra bikarbonattransport man får vid pågående syrasekretion hjälper till att bibehålla neutralt pH vid ytepitecellerna. Därmed skyddas magslekhinnan bättre och slemhinnan acidifieras i mindre grad, vilket har en mindre blodflödesökning och opåverkad permeabilitet som följd.

**II. Acid transport through channels in the mucous layer of rat stomach.**


Hur syran som produceras i magkörtlarna tar sig till maglumen utan att surgöra slemlagret och förstöra pH-gradienten har länge diskuterats. Ett oscillerande tryck i magkörtlarna har tidigare uppmätts, vilket höjs vid stimulerad syrasekretion. Vår hypotes var att detta körteltryck skjuter ut syran i slemlagret i speciella kanaler, och att syratransport från körteln till maglumen sker i enbart dessa kanaler.

Vid spontan syrasekretion infärgades blå punkter i slemmet just ovanför kryptöppningarna och trådlika strukturer ”kanaler” (5-7µm i diameter) som ledde från dessa punkter vid kryptöppningarna mot maglumen. Slemmet mellan kryptöppningarna blåfärgades inte. När syrasekretionen stimulerades, blåfärgades det översta skiktet av slemlagret. Om detta togs bort, var resterande slem ofärgat, vilket verkar tyda på att indikatorfärgen faller ut vid lågt pH. När Congorött lades på igen, blåfärgades körtelöppningar och kanaler i det inre slemlagret. När syrasekretion tillfälligt hämmades färgades kryptöppningarna med dess trådlika kanal röda istället. Om syrasekretionen totalt hämmades i en vecka, så att slemlagret hunnit nybildas, såg inga kanaler, dock röda punkter vid kryptöppningarna.

Dessa resultat visar att syran penetrerar slemlagret enbart i speciella ”kanaler” och att slemmet mellan dessa inte surgörs, vilket vi i studie (I) kunde mäta som en pH-gradient under pågående syrasekretion.

När vi tog bort det övre slemlagret vid pågående syrasekretion, kunde en tillväxt av det övre slemlagret mätas. Så skedde inte när syrasekretionen hade varit totalt hämmad i en vecka och inga ”kanaler” fanns. Detta skulle kunna betyda att det yttre slemlagret till stor del består av slem som produceras nere i körteln och som tar sig igenom det inre lagret i ”kanalerna”.

Efter att ha separerat de båda slemlagren visade vi spektrofotometriskt att Congorött adhererade till större grad till det yttre slemlagret än det inre. Detta kan tyda på att slemmet som utgör det yttre slemlagret bildas nere i körtlarna och transporterades genom det inre slemlagret i ”kanalerna”.

I denna studie har vi lyckats påvisa ”kanaler” i slemlagret, vilka börjar vid kryptöppningarna och leder mot maglumen. Syratransporten från körteln till lumen sker enbart i dessa ”kanaler”. De bildas troligen med hjälp av det
höga körteltrycket och verkar bestå av det slem som produceras nere i körteln.

III. The importance of mucus layers and bicarbonate transport in preservation of gastric juxtamucosal pH.

Phillipson M, Atuma C, Henriksnäs J & Holm L.

I denna studie undersöktes hur viktigt det yttre slemlagret var för upprätthållandet av pH vid ytepitelcellerna (pHjm), med stimulerad eller inhiberad syrasekretion och pH 1 i lumen. Dessutom studerades påverkan av prostaglandin-stimulerade bikarbonatsekretion på pHjm, samt hur bikarbonat transporteras över ytepitelcellerna.

I Inactin®-sövda råttor frilades magsäckens korpusdel, monterades med slemhinnan uppåt för intravitalmikroskopi och täcktes av varm fysiologisk koksaltlösning. pH-känsliga mikroelektroder, förbundna med en mikromanipulator, fördes in i slemmet med en vinkel på ca 30° mot slemhinnan. I hälften av försöken sögs det yttre slemlagret försiktigt bort precis innan pH 1 lades på luminalt. Syrasekretionen stimulerades med pentagastrin, inhiberades med ranitidin och prostaglandinsyntesen hämmades med indomethacin, alla givet intravenöst. DIDS gavs luminalt och hämmar Cl-/HCO₃⁻ transportören som sitter på apikala membranet av ytepitelcellerna.

Som visats i studie (I), sjönk pHjm, kraftigt av pH 1 luminalt om ingen syrasekretion pågick. Om syrasekretionen stimulerades erhölls enbart en knappt sänkt pHjm, av pH 1 i lumen. Avlägsnadet av det yttre slemlagret påverkade inte pHjm, vid luminal pH 1 vare sig syrasekretionen var stimulerad eller inhiberad. När syrasekretionen stimulerades påverkade inte hämmandet av prostaglandin-syntesen pHjm, oavsett om det yttre slemlagret tagits bort eller ej. Alltså spelar inte den prostaglandin-beroende bikarbonatsekretionen en stor roll i neutralisering av tillbakadiffunderad syra under pågående syrasekretion. När både syrasekretionen och prostaglandinsyntesen hämmades, sjönk pHjm, lite under pH 1 i lumen men framför allt skedde återetableringen av ett neutralt pHjm långsammare om det yttre slemlagret tagits bort. Detta tyder på att det yttre slemlagret är viktigt för pHjm, enbart om både syrasekretion och prostaglandinsyntesen hämmats. Om magslemhinnan i syrastimulerade djur förbehandlades med DIDS sjönk pHjm kraftigt vid administrering av luminal syra pH 1. Detta visar att den
bikarbonat som neutraliserar tillbaka diffunderad syra i slemmet transporteras över yttepitelcellerna genom en DIDS-känslig transportör.


IV. Inducible Nitric Oxide Synthase is involved in the acid induced gastric hyperemia in rats and mice.

Phillipson M, Henriksnäs J, Holstad M, Sandler S & Holm L. Accepted in Am J Physiol Gastrointest Liver Physiol 2003

När magslemhinnan utsätts för stark syra i kombination med alkohol, ökar mukosablodflödet och sår uppkommer. Denna blodflödesökning har visat sig bero på frisättning av kväveoxid (NO). NO produceras av tre olika sorts enzym som namngets efter den plats de först hittades på. Två av enzymen uttrycks konstitutivt och kallas eNOS (endotel NOS) och nNOS (neuronalt NOS). iNOS (inducerat NOS) uttrycks i makrofager efter induktion vid tex inflammation. I studie (I) fann vi att mukosablodflödet ökade av enbart luminal syra, och inga sår uppstod. I den här studien undersökte vi om även denna blodflödesökning beror av NO och vilket NO-producerande enzym som är inblandat.

Magslemhinnan i Inaktin®-sövda råttor eller isofluran-sövda möss frilades och monterades med korpusdelens sleminna uppåt. Blodflödet mättes i både råttor och möss med laser–Doppler flödesmetri (LDF), som ger ett mått på procentuell förändring i blodflödet jämfört med en kontrollnivå. Permeabiliteten uppskattades i råttor med hjälp av clearance av ⁵¹Cr-EDTA, som givits intravenöst, från blod till maglumen. Efter en stabil kontrollperiod lades 155mM HCl på magslemhinnan i två tio-minuters perioder. Syran sköljdes noggrant bort och försöket fortsatte i ytterligare trettio minuter. Till råttorna gavs L-NNA (ospecifik NOS inhibering), L-NIL (specifik iNOS inhibering) eller SMTC (specifik nNOS inhibering) intravenöst. Genetiskt manipulerade möss där antingen iNOS eller nNOS deaktiverats (iNOS⁻/⁻ och nNOS⁻/⁻) jämfördes med kontrolldjur. Magslemhinnan hos möss skrapades och iNOS mRNA innehållet bestämdes med reallids RT-PCR.
Luminal syra gav en signifikant ökning av mukosablodflödet hos kontrolldjuren. Om råttorna förbehandlats med L-NNA, L-NIL eller SMTC försvann denna syrainducerade hyperemi. Både L-NNA och SMTC gav en blodtrycksökning, via hämning av eNOS, vilket tyder på att SMTC inte specifikt hämmar nNOS. Magslemhinnans permeabilitet ökade av luminal syra men återvände direkt när syran sköljts bort, vilket visar att 155mM HCl inte permanent skadar slemhinnan. I iNOS-/- erhölls ingen blodflödesökning av luminal syra, medan hyperemin kvarstod opåverkad i nNOS-/-.. Då blodflödesökningen av luminal syra måste anses vara en normalt förekommande försvarsmekanism visar dessa resultat en inblandning av iNOS i normalfysiologin av magslemhinnan. Vi fann även konstanta nivåer av mRNA för iNOS i slemhinnan av möss i mängder som var oberoende av anestesi, operation eller luminal syra.

Sammanfattningsvis visar denna studie en ny funktion av iNOS i magslemhinnans skydd mot syra då iNOS är inblandad i syrainducerad hyperemi och verkar vara konstitutivt uttryckt.