PARIE TAL CELL REGENERATION IN
RAT GASTRIC MUCOSAL WOUNDS

A quantitative light and electron microscopical study

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ABSTRACT

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A quantitative light and electron microscopical study

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The aims of the study were to obtain a method with which it would be possible to produce standardized wounds in the gastric mucosa, and to follow the regeneration of the acid producing parietal cells in those lesions during different experimental conditions. Quantitative methods applied to light and electron microscopy were used.

Wounds were cauterized in the corpus mucosa in Sprague-Dawley rats and in addition, pyloroplasty, truncal vagotomy with pyloroplasty or antrectomy were performed. Other groups of rats with wounds were given long-term treatment with pentagastrin or cimetidine. Stimulation tests were carried out in two groups of wound operated rats.

After different periods of time the animals were perfusion fixed and specimens from the wounds and normal mucosa beside the wounds were prepared for light and electron microscopy. By means of stereological techniques, different mucosal and cellular structures were then measured.

Parietal cells were found in 90 days old wounds. At this stage they were immature with large nuclei and few specialized cell organelles. In spite of this appearance they were able to respond morphologically to stimulation and to secrete acid. With further healing the morphology of the parietal cells became normal, but their volume fraction in the mucosa remained subnormal. The fraction of mucosa occupied by epithelial cells also stayed lower than normal.

Pyloroplasty resulted in decreased cell and nuclear size of both normal and regenerating parietal cells. In the latter, there was also a decrease in the mitochondrial volume density. If a truncal vagotomy was added to the pyloroplasty these changes disappeared and, in addition, an increase in parietal cell volume density was noticed in the normal mucosa.

Antrectomy produced smaller parietal cells, and their maturation was delayed. Furthermore, mucosal thickness decreased. If pentagastrin was given to rats with wounds an increase in the number of parietal cells was noted, but maturation and morphology remained unaffected.

Cimetidine treatment did not affect the parietal cell volume density in wounds or normal mucosa. However, a large increase in the secretory surface density was noticed when the effect of the last dose had ceased.

Key words: Rat, gastric mucosa, gastric wounds, parietal cells, regeneration, pyloroplasty, truncal vagotomy, antrectomy, pentagastrin, cimetidine, carbacholine, stereology, light microscopy, electron microscopy, pH-studies.
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To My Father ...

Why is my wound incurable which refuseth to be healed ...

Jer. 15:18
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REPORTS CONSTITUTING THE THESIS

This thesis is based on the following publications and manuscripts, which will be referred to by their Roman numerals:


II. Blom H.: Immature parietal cells in healing gastric wounds respond to stimulation. An experimental study in the rat. Digestion, in press.


IV. Blom H. and Erikoinen T.: Is there a trophic effect of pentagastirin on regenerating parietal cells in rat gastric mucosal wounds? (Submitted for publication).

Experimental ulcer studies

In 1586 Donati published the first clear reports on the gastric ulcer disease (1). Two centuries later Abercrombie (2) and Cruveilhier (3) described the gastric lesions, and they also suggested causative factors. At this time, different models for studying ulcer etiology and healing began to appear (4), and in 1875 Quincke (5) described three possible ways to produce gastric wounds in dogs, viz. by pressure, by ligation of vessels, and by heat applied by hot irons.

Experimental ulcers* have since been produced in many different ways (6), partly depending on the aims of the study. Thus, if the interest has been focused on drugs and/or surgical procedures which can prevent the appearance of mucosal lesions, methods have been chosen which result in a consistently high incidence of readily discernible ulcers. Such methods include ligation of the pylorus in rats (the so called Shay-rat, 7), different kinds of stress (8 - 12), and administration of various ulcerogenic drugs, such as histamine (13 - 14), reserpine (15 - 17), adrenaline (18 - 19), cortisone (20 - 23), and other antiinflammatory drugs (16, 24 - 28).

All these methods have in common that they produce superficial erosions (29), seldom reaching below the muscularis mucosae, rather than deep penetrating "chronic" lesions resembling human gastric ulcers (30 - 32). The number of these superficial lesions and their exact location cannot be predicted.

* The term ulcer refers to tissue lesions which arise from the action of corrosive agents, whereas wounds result from mechanical trauma. In experimental research on gastric lesions, mechanical methods of various kinds (vide infra) are often employed to produce the primary wound in the mucosa. However, the subsequent development and ultimate healing of such wounds are obviously influenced by the gastric juice, including its corrosive acid. Against this background it is not entirely obvious whether the term gastric wound or ulcer should be preferred. Thus, in this publication the terms have been used in parallel.
Similar erosions can also be produced by stimulation and/or injuring of the brain, predominantly the hypothalamus (for review see 33).

Thus, if these procedures are appropriate when one is interested in studying how to prevent the occurrence of mucosal damage, they may be less suitable if ulcer healing tests are to be performed, mainly because they cannot be standardized with respect to localization and depth.

Since most therapy concerns healing of an already existing lesion, attempts have been made to produce standardized wounds which morphologically resemble human chronic ulcers. Such wounds can be obtained in many different ways, but the most common procedure is probably surgical excision of parts of the mucosa, usually down to, or including, the muscularis mucosae (34 - 40). Cauterization by hot irons (41 - 46) and ligation of blood vessels (47) have also been employed. In 1969 Takagi et al. (48) described a method "for production of chronic gastric ulcers" by injections of acetic acid into the subserosal layers of the glandular mucosa, and this procedure has later been modified and used also by others (49 - 51). Recent models include photocoagulation by laser (52 - 53) and freezing of the mucosa by means of a cryoprobe (54).

In most ulcer healing studies the principal interest has been to determine how long time it takes until the wound is covered by epithelium and how this time can be affected. Less interest has been devoted to the events occurring on the cellular, or subcellular, level during regeneration. The majority of studies published on this subject are based on observations made by light microscopy (34 - 38, 40 - 47, 49, 53 - 54) and only a few investigators have utilized the electron microscope (39, 55 - 56).

At the light microscopical level it is difficult to differentiate the epithelial cell types in the gastric mucosa with accuracy, and since regenerating cells in a healing wound differ morphologically from their normal counterparts, light microscopical identification becomes uncer-
tain. This fact probably contributes to the divergent opinions expressed by different authors, e.g. with regard to the parietal cells. Thus, some investigators believe that parietal cells are present in healed ulcers (34, 36 - 39, 42, 44, 47, 56), while others claim that these cells never reappear (35, 41, 46, 54).

In previous ulcer healing studies no attempts have been made to quantitate the regeneration of the different epithelial cell types, and consequently it has not been possible to decide when a lesion is "completely healed". Furthermore, up to date no systematic information has been available on the morphological events occurring within the cells in healing gastric wounds.

The parietal cells
The parietal cells - sometimes referred to as the oxyntic (from the Greek oxys-acid) cells - are found mainly in the corpus, or fundic, mucosa in the mammalian stomach (57). The cells are relatively large, in the rat the volume has been calculated to about 1100 μm³ (58) and in man to 5500 μm³ (59), and they are ellipsoidal or conical in shape. They are situated peripherally along the gland tubules (parietal position), resulting in knob-like protrusions (60). They are characterized by a system of secretory canaliculi (Fig. 1), which were first described by Golgi in 1893 (61). The intracellular location of these canaliculi was established by Zimmermann in 1898 (62).

A remarkably high amount of mitochondria, occupying between 22 to 47% of the cytoplasmic volume (58, 63 - 66), with densely packed cristae suggests a high oxidative metabolism in the parietal cells (57) and this feature has been utilized for histochemical identification (46). Thus, under normal conditions the succinic dehydrogenase (SD) activity is much higher in the parietal cells than in any other epithelial cell type in the gastric mucosa (67).
Fig. 1. Mature parietal cell from a starved rat. The cell is conical in shape with the nucleus (N) situated peripherally. There is an abundance of mitochondria (M) and in between these organelles many tubulo-vesicles (arrow) are seen. The cell is not stimulated, hence, the intracellular canalicuar system (C) is poorly developed.
Another characteristic feature is the presence of a varying amount of cytoplasmic tubules and/or vesicles, often referred to as tubulo-vesicles (57). The tubulo-vesicles and the intracellular canalicular system are intimately involved in the secretory process of the parietal cells.

Since the secretory product is hydrochloric acid (68), the parietal cells are directly involved in the etiology of the peptic ulcer disease, and many studies have been carried out to clarify the secretory process and how it can be modified. Morphological investigations reveal that stimulation results in an increase in the area of the membrane limiting the intracellular canaliculi - the secretory surface - at which acid probably is produced (68). This increase is paralleled by a decrease in the area of the tubulo-vesicles (69 - 71). When stimulation ceases the cell returns to its resting state with many tubulo-vesicles and a relatively small secretory surface area (70 - 71). Based on quantitative data it has been postulated that the tubulo-vesicular membranes are reversibly incorporated into the canalicular membrane during stimulation (72).

Various drugs, for example the H₂-receptor antagonist cimetidine, as well as certain surgical procedures, viz. vagotomy and antrectomy, can diminish acid secretion. However, surprisingly few studies have been devoted to the morphological consequences of these treatments and available data are contradictory.

Thus, some authors have failed to demonstrate any significant effects of vagotomy on the parietal cell mass (73), while others have seen transient (74 - 75) or chronic (76) changes.

On the other hand, most investigators agree that antrectomy results in hypoplasia of the parietal cell mass. This is probably an effect of decreased serum gastrin levels which is a consequence of an antrectomy (77 - 78). Since gastrin is known to exert a trophic effect on the gastric mucosa (78) decreased levels thus results in hypoplasia of the gastrointestinal epithelia (79).
Intracellular changes are minimal in normal parietal cells after vagotomy or antrectomy (58), and very little is known about possible effects on regenerating cells.

In the present series of studies, a standardized method for the production of chronic gastric wounds has been developed, and the regeneration of the parietal cells has been followed by means of quantitative methods applied to light and electron microscopy. The effects of surgical and pharmacological interference have been investigated.

AIMS OF THE STUDY

Against the background outlined above the specific aims of the present study can be summarized as:

- to develop an ulcer model with which it should be possible to follow the regeneration of epithelial cells in the gastric mucosa under controlled conditions by means of quantitative techniques applied to light and electron microscopy

- to follow the regeneration of the acid producing parietal cells under normal conditions

- to investigate whether regenerating parietal cells are capable to respond to stimulation

- to investigate if truncal vagotomy with pyloroplasty or antrectomy affect parietal cell regeneration and/or morphology

- to investigate if pentagastrin or cimetidine administration affect the parietal cell regeneration and/or morphology.
MATERIALS AND METHODS

Animals
Male Sprague-Dawley rats (Anticimex, Sweden) were used for the investigations. At the beginning of the experiments they were two to three months old and weighing about 300 g. During the experimental period they were kept separate in normal cages, with free access to water and a standard laboratory rat diet (ASTRA-EWOS, Sweden).

Operations
Before surgery the animals were fasted for 24 hours in wire-mesh bottom cages with free access to water. After anesthesia with sodium pentobarbital (40 mg/kg) they were subjected to one or more of the following operations:

Cauterization of wounds (I - V). Using a modified hemostat (I) a wound, 1.6 cm² in size, was cauterized in the anterior gastric wall about 3 mm distal to the transverse ridge. One of the shanks of the instrument was heated in boiling water for two minutes. During this time the stomach in the laparotomized animal was turned inside out through an incision in the rumen. After cooling in room temperature for 20 seconds the hemostat was locked over the gastric wall with the hot shank in contact with the mucosa. The initial temperature during cauterization was 70°C and the pressure applied over the gastric wall was constant. After one minute the instrument was removed, and to facilitate recognition two silk sutures (6 - 0) were put in the wound edges. The ruminal incision was then closed and eventual further surgery was carried out.

Pyloroplasty (III). Pyloroplasty (58, 80) was performed by making a longitudinal incision, about 7 mm long, in the pyloro-duodenal junction and closing the opening transversally with silk sutures (Fig. 2A). This operation prevents the gastric stasis which follows truncal vagotomy (81).
Fig. 2. Schematic drawings presenting the different operations performed in addition to cauterization. A: pyloroplasty with truncal vagotomy. B: antrectomy with gastroduodenostomy (ad modum Billroth I).
Truncal vagotomy (III). Both vagal trunks were cut and about 1 cm of them was removed (82). To eliminate any remaining nerve fibers, the muscle layers of the esophagus were also cut just above the cardia (58) (Fig. 2A). The completeness of the vagotomy was checked light microscopically in paraffin embedded tissue taken at the time of sacrifice. Pyloroplasty was always performed together with the vagotomy.

Antrectomy (III). The distal part of the stomach and the first 5 mm of the duodenum were removed. The resected portion of the stomach was limited by a line from the cardia on the lesser curvature to halfway between the pylorus and the rumen on the greater curvature. Gastrointestinal continuity was reestablished by an end to end gastroduodenostomy (83) (Fig. 2B). Care was taken not to disturb the arterial or nerve supply to the gastric remnant.

Sham operations (I). One group of animals was sham operated. The abdomen was incised and the stomach opened through the rumen. The mucosa was then exposed by turning the stomach inside out. After one minute the wounds were sutured.

Postoperative care
The antrectomized animals were fasted also after operation, and during this time they received daily subcutaneous injections with 10 ml of Tyrode solution. After three days these rats were put on normal diet. All the other operated animals were returned to cages, and allowed food and water immediately after operation.

During the entire experimental period all rats were weighed weekly, and if there were any signs of abnormal weight increase they were discarded.

Drug administration
Cimetidine (V). The cimetidine (Smith, Kline & French, no. 92334) was dissolved in 1 M HCl. Water was then added to make the drug concentration 25 mg/ml and the pH was adjusted to around 6 with 0.1 M NaOH. The
solution was administered orally twice daily (at 8 am and 5 pm) through a stomach tube, and the dose was 75 mg/kg of cimetidine each time (84 - 86). The treatment continued for 130 days. As controls, one group of rats was given water.

Pentagastrin (IV). Pentagastrin (Peptavlon\textsuperscript{R} ICI) was administered as subcutaneous injections twice daily. The hormone, 250 µg/kg (87), was mixed with 16% hydrolyzed gelatin (Calcitonin Diluent B, prepared by Armour Pharmaceutical Co.) immediately before injection. A control group received the gelatin diluted with physiological saline.

Gastrin analyses were performed on blood samples taken from the animals immediately before tissue fixation. The blood was centrifuged and the plasma freeze-dried. The gastrin assays were then performed by Dr. J.F. Rehfeld, Århus, Denmark.

Stimulation tests
Morphological studies (II). Rats with 90 days old wounds, fasted for 24 hours, were used. The animals were put into Bollman cages and two catheters were placed subcutaneously. Pentagastrin, 20 nmol/kg, h, and carbacholine, 110 nmol/kg,h (Helander & Sundell, to be published) or Ringer's solution was infused continuously during one hour. After this time the animals were rapidly anesthetized with ether, and the stomachs fixed as to be described.

pH measurements. (II). Animals with 90 days old wounds were stimulated as above and were then anesthetized with ether. The stomach was opened and the mucosa exposed through a ruminal incision. The wound was localized and wiped once with a dry cotton stick. Small chips of pH-paper (Pehanon, Fluka AG) were then put on the mucosal surface in the wound, wound edge and dorsal gastric wall. The pH was registered when the pH-paper, which had a specific colour for each pH-interval, was moistened.
**Tissue preparation**

**Fixation procedures (I - V).** All animals were fasted in wire mesh bottom cages for 24 hours before sacrifice. Under anesthesia the abdomen was cut open and the aorta exposed. A metal cannula, connected to a peristaltic pump, was introduced into the vessel and a rinsing solution, a modified Tyrode buffer (88), was pumped into the vascular system. At the same time the portal vein was opened, the intestines tied off, and the aorta above the diaphragm clamped. When the perfused tissue appeared empty of blood, perfusion was continued with fixative. At the same time 10 ml of the same solution was injected directly into the stomach.

The fixative consisted of a mixture of 4% glutaraldehyde, 3% formaldehyde, and 0.05% trinitrophenol in a 0.05 M Na-cacodylate buffer (89).

After perfusion for 10 minutes the stomach was cut open and, under a dissection microscope, specimens were taken from the center of the wound and from a corresponding site in the dorsal gastric wall. Fixation by immersion was then continued for two hours in the same fixative. After rinsing in 0.1 M Na-cacodylate buffer, postfixation was carried out in 1% OsO₄ in 0.1 M Na-cacodylate for two hours. All solutions were kept at room temperature.

Dehydration was performed in rising concentrations of ethanol and embedding in Epon 812.

**Specimens for light microscopy.** Survey sections of whole wounds were made from paraffin embedded tissues (I, III), fixed in 10% neutral formalin. Such sections were stained with hematoxylin-eosin or PAS. For stereological investigations (IV, V), 1 μm thick epon sections contrasted with toluidine blue were used.

**Specimens for electron microscopy.** Sections, 60 - 80 nm thick, were cut in a Porter Blum MT2-B microtome and put on formvar coated one hole copper grids. Section thickness was estimated by the method described
by Small (90). After contrasting with uranyl acetate and lead hydroxide the sections were studied in a Philips EM 300 or a JEOL JEM-100CX electron microscope.

**Stereological analyses**

**Light microscopical analyses (IV, V).** Stereological analyses were carried out using oil immersion optics at 1,000 times magnification. The technically best section from each tissue block was used and in each such section two randomly chosen areas were analyzed. The analyses were performed by means of an occular square grid with about 9,000 test points per mm$^2$ of tissue section. The points falling over parietal cells, epithelial cells and whole mucosa (excluding the lamina propria), respectively, were counted. The parietal cell volume density in the mucosa ($V_{\text{Vpc}}$), i.e. the fraction of mucosa taken up by parietal cells, was calculated according to methods adopted from Weibel (91). The volume density of epithelial cells in the mucosa ($V_{\text{Vec}}$) was also assessed.

Using an eye-piece micrometer the mucosal height was measured in sections which were cut parallel to the gastric glands.

**Electron microscopical analyses (I - V).** The technically best section on the grid was chosen and photographed at a magnification of about 600 times. On each survey, an area limited by two parallel lines about 50 - 250 μm apart, the mucosal surface, and the bottom of the glands was randomly chosen (Fig. 3). Within such an area all parietal cells showing their nucleus were photographed at a primary magnification of about x 5,000. Paper prints of these cells (final magnification around x 11,000 - 15,000) were coded at random and subjected to stereological analyses.

A multipurpose test grid (91) (Fig. 4) was superimposed over the parietal cells and the number of points falling over the cell, nucleus, and mitochondria, respectively, were counted. The number of intersection points between the test line and the secretory surface (I - V) and the tubulo-vesicular surface membranes (I) were also counted.
3. Survey micrograph of perfusion fixed normal gastric mucosa. Between the gastric glands (GL) and pits (GP) a relatively extensive lamina propria (LP) is seen.
Using these figures the mitochondrial volume density ($V_{Vm}$) was calculated in relation to cytoplasmic volume (excluding the nucleus), and the nuclear volume density ($V_{Vn}$) in relation to cell volume. The profile areas of the nuclei and the cells were also calculated according to the formula:

$$A = \left(\frac{d}{M}\right)^2 \times \sqrt{\frac{3}{4}}$$

where $A$ is the profile area ($\mu m^2$), $d$ the length of the short line segment on the test grid (Fig. 4), and $M$ the magnification.
The surface densities \((m^2/cm^3\) of cell volume) of the canicular (I, II, IV and V) and tubulo-vesicular (I) membranes were calculated as follows:

\[
L = \frac{P \times d}{2 \times M}
\]  

(1.2)

\(L\) is the length of the test line and \(P\) the number of test points falling over the cell profile. The surface density, \(S_V\), can then be calculated according to:

\[
S_V = \frac{2 \cdot I}{L}
\]  

(1.3)

where \(I\) is the number of intersection points between the test line and the surface measured.

Thus, (1.2) and (1.3) gives:

\[
S_V = \frac{4 \times I \times M}{P \times d}
\]  

(1.4)

Correction of systematic errors connected with the stereological methods

"Holmes effect". Stereological formulas hold without restrictions only when the sections studied are infinitely thin. What is really investigated in tissue sections is the projection into an observation plane of the entire content of a relatively thick slice (in the present studies between 50 to 80 \(\mu\)m). This was recognized by Holmes in 1927 (92) (Fig. 5), and he suggested formulas to compensate for the overestimation of the volume density of the profiles measured.

If corrections for the "Holmes effect" (91, 93) are applied on the data in Paper I one finds that the mitochondrial volume density might have been overestimated by a factor of between 13 to 24\% (depending on section thickness), and the secretory surface density by some 25 to 35\%.
It should be pointed out that these corrections for the "Holmes effect" are valid only when the measured structures have a higher density than the surrounding structures. This is the case for mitochondria and surface membranes.

Fig. 5. If a relative thick section (C) is cut from the specimen (B) the opaque structures will be overestimated due to projection of slice content (Holmes effect). This overestimation decreases when the sections becomes thinner (A). (After Weibel and Elias).

Nuclear volume density overestimation. To eliminate difficulties in identification, only such cells which exposed the nucleus were analysed. This procedure results in overestimation of the nuclear volume density. The error due to this biased sampling procedure has been analysed for spherical cells by Konvinski & Kozlowski (94), and using their methods it can be calculated that an experimentally found nuclear volume density of e.g. 14.5% (I) should be corrected to 9.2%. However, since the parietal cells are not spherical in shape, but rather conical, it is doubtful whether this correction is appropriate.
For more detailed information on the stereological principles, techniques, and theories see references 91 and 95.

**Errors due to tissue preparation.** Fixation, dehydration, embedding and sectioning change the morphology of all cell types (96). No attempts have been made to correct for these changes.

The stereological methods are thus hampered by several systematic errors. None of these have been corrected for in the present studies and therefore the reader should pay more attention to **comparisons between** the different groups of data than to the absolute figures. The values are comparable since all tissue have been processed in exactly the same way.

**Personal errors.** To estimate the personal error of the method, duplicate measurements were made within a six-month interval. This was done on all measured parameters in one rat in Papers I and III. The error in the relation to the mean values amounted to between 1 - 3% for the cell profile area, 2 - 2.5% for the nuclear profile area, 2 - 2.5% for the nuclear volume density, 3 - 9% for the mitochondrial volume density, and 7 and 9% for the secretory and tubulo-vesicular surface densities, respectively.

**Statistical analysis**
Significance of differences between mean values was calculated using the two-tailed Students t-test.
RESULTS

Animal recovery after the different surgical procedures
The animals generally withstood the surgical interventions well. When an ulcer operation, with or without pyloroplasty (I - V), or a sham operation (I, III) was performed there was no mortality at all. However, in the vagotomy group (III) the mortality was rather high, about 35%, with most deaths occurring late, usually more than 90 days post-operatively. The mortality among the antrectomized rats (III) was also considerable, but in this group most deaths occurred on the first post-operative days.

All animals were weighed weakly but no significant difference was noted in the initial and final weights between the different groups with one exception, the cimetidine treated rats (V), which increased more than the corresponding control animals.

The rats used in the cimetidine study (V) were orally incubated twice daily. As a rule this treatment was well tolerated.

In the pentagastrin study (IV) the animals received subcutaneous injections twice daily for 90 days. No cutaneous infections or other adverse reactions were seen due to this procedure.

Normal regeneration (I)
The mucosa. Examination of the wounds 24 hours postoperatively, revealed a lesion which corresponded in size to the cauterized area, and the entire mucosa, including the muscularis mucosae was coagulated. After one week there was still a large mucosal defect in the center of the wound, but in the periphery a layer of cuboidal to low columnar cells containing a varying amount of mucous, PAS-positive granules were seen.

After 30 days of healing the entire defect was covered by a mucosa forming pylorus-like glands, and at this stage the most common cell
type was a poorly differentiated "surface mucous cell". With further healing the mucosa became more similar to that found in the normal corpus region. However, the percentage of mucosa taken up by epithelial cells never reached normal levels (Fig. 6).

![Graph](image)

**Fig. 6.** Graphic illustration of the epithelial and parietal cell regeneration in the gastric wounds (mean ± S.D.). Open circles: epithelial cell volume density (% of mucosal volume, not including the muscularis mucosae). Filled circles: parietal cell volume density (% of epithelial cell volume). The horizontal lines represent normal mean values ± S.D.

Parietal cells were found after 90 days of healing, but the total volume of these cells remained subnormal (Fig. 6).
The parietal cell. The first parietal cells, positively identified by the presence of intracellular canaliculi, were immature in appearance with large nuclei, low mitochondrial volume density and a small total amount of intracellular membranes. The latter is the sum of the secretory surface and tubulo-vesicular surface membrane densities, and the low value in these parietal cells was mainly due to a decrease in the tubulo-vesicular surface density.

After 260 days, all ultrastructural peculiarities seen in the early "wound parietal cells" had disappeared.

The response to stimulation in normally regenerating parietal cells (II)

The purpose with this study was to investigate, whether regenerating parietal cells in the wounds responded to stimulation. One group of unstimulated rats was compared with a group which had been stimulated.

After stimulation with pentagastrin and carbacholine, at doses which normally stimulate the parietal cells to near maximal acid secretion, there was a large increase in the secretory surface area in both normal and regenerating parietal cells, the increase being 90% and 76% respectively.

Moreover, stimulation resulted in a decrease in pH on the mucosal surface of the wounds and the normal mucosa beside the wounds. On the elevated margin surrounding the wounds the pH was high, usually between 4 and 9, and did not change with stimulation.

Effects of pyloroplasty, pyloroplasty in combination with truncal vagotomy, and antrectomy (III)

Pyloroplasty resulted in changes in both healing and normal mucosa. In the wounds, smaller parietal cells with smaller nuclei and lower mitochondrial volume density were seen. In normal mucosa pyloroplasty tended to decrease the size of the parietal cells. A decrease in nuclear profile area was also noted.
In rats subjected to truncal vagotomy in addition to pyloroplasty, the parietal cells from normal mucosa were significantly larger than cells from the corresponding pyloroplasty group. When compared with parietal cells from sham operated animals no ultrastructural changes were seen after 130 days regeneration.

However, truncal vagotomy plus pyloroplasty resulted in marked increase in the parietal cell volume density in normal mucosa, both when compared to sham operated and pyloroplasty operated animals.

In the wounds, truncal vagotomy plus pyloroplasty did not cause any alterations in the morphology of the parietal cells in addition to those found after pyloroplasty alone.

The antrectomy, by which the entire antrum (and thereby most of the gastrin producing cells) was removed produced generalized changes in both regenerating and normal mucosa.

In the wounds there was a decrease in mucosal height and in parietal cell size. Furthermore, there was a marked decrease in nuclear size and mitochondrial volume density.

In normal mucosa, antrectomy resulted in similar changes as those seen in the wounds. Thus, a decrease in mucosal height was noted in addition to a decreased cell and nuclear size. Furthermore, a decrease in epithelial cell volume density was noted.

Effects of long-term administration of pentagastrin (IV)

Injections with pentagastrin, twice daily for 90 days, at a dose of 250 μg/kg resulted in marked changes in the parietal cell mass both in wounds and in normal gastric mucosa. At both sites, hyperplasia was seen, and in the wounds there was a 150% increase in the parietal cell volume density. In normal mucosa the increase was 30%.
No changes were observed in mucosal height or in parietal cell size and therefore the increase in parietal cell volume density must be the result of an increased number of cells.

Pentagastrin did not affect parietal cell maturation or ultrastructure during the experimental period.

**Effects of long-term treatment with cimetidine (V)**

The dose of cimetidine used in this study reduces basal acid secretion to nearly nil (97). The last dose was given 15 to 17 hours prior to fixation of the tissue, and at this time the inhibiting effect of this dose would have ceased (84, 98 - 99). In the light microscope no alterations were observed in the parietal cell mass in the cimetidine treated group compared to the group of rats which had received water only. However, the regenerating mucosa in the cimetidine treated rats was significantly thicker than in the control group.

The ultrastructure of the parietal cells differed between the two groups in one important aspect, viz. the secretory surface density. The intracellular canalicular system in parietal cells from animals which had been given cimetidine was more extensive than in cells from control rats. Thus, there was an increase by 76% in regenerating parietal cells and by 49% in parietal cells from the normal (dorsal gastric wall) mucosa.

The plasma gastrin levels at the time of tissue fixation was not significantly different between the two groups.
DISCUSSION

The ulcer model
Depending on the aim, different procedures are used to induce wounds in the gastric mucosa (7 - 54). In the present investigations the aim was to develop a model by which the regeneration of parietal cells could be followed under controlled conditions. Furthermore, the morphology of the wounds should as close as possible resemble the chronic gastric ulcer in man. Such a wound is characterized by deep penetration, below the muscularis mucosae, and a relatively slow healing rate (30, 43).

Many methods can be used to obtain such wounds (40 - 45, 47 - 48, 54) but, the standardization has often proved to be a major obstacle.

With the cauterization method used here the lesions were of the same size and depth every time. This could be achieved since the two factors that contributed to the genesis of the lesions, the heat and the pressure applied by the hemostat, were kept constant.

However, it must be emphasized that this is an artificial lesion, and the etiological factors contributing to a clinical ulcer are thus missing. On the other hand, it can be assumed that in the absence of such factors, the conditions for regeneration will probably be optimal, and the wounds should thus permit detailed observations, under controlled conditions, on the healing process, also at a subcellular level.

Another point of dispute might be the localization of the mucosal lesion. In man, a benign gastric ulcer is preferentially located to the antrum-corpus border on the lesser curvature (31) and not in the corpus region as in this study. However, the main purpose of the present investigation was to follow the parietal cell regeneration and therefore an area was chosen where the parietal cell density is maximal (100).

In the present investigation rats were used as experimental animals. There are many differences, both anatomically and functionally, between
the human and the rat stomach, but the parietal cells in these organs are quite similar in appearance. The rat parietal cells are smaller (58, 59) and contain a smaller volume of mitochondria (58) compared to human parietal cells (101) but otherwise there are no major differences neither in the resting (102) or stimulated state (69, 103).

**Tissue preparation**

Considerable experimentation proceeded the choice of fixative and finally a modified Bouin solution containing glutaraldehyde, formaldehyde, and picric acid (89) was chosen because of its superior ability to preserve the ultrastructure of both normal (63) and regenerating parietal cells.

This fixative has a very high total osmolarity, around 1,400 mOsm/kg and considerable shrinkage of the tissue might be expected. However, when the data obtained from parietal cells preserved with this fixative were compared with data from parietal cells fixed at lower total osmolarity, around 470 mOsm/kg (58), the only difference seen was a larger tubulo-vesicular surface density in the latter group. This was not surprising since these structures are very sensitive to alterations in tonicity. Thus, hypotonic solutions produce vesicular structures whereas an increase in osmolarity gradually results in a predominance of tubules (104).

It would be possible to calculate the degree of shrinkage/swelling of a processed tissue if a value on the "true" in vivo conditions could be obtained. For the gastric mucosa such a parameter has been calculated by Davenport (105) who estimated the sodium space in the oxyntic mucosa in dogs. This space, which was 38 to 47% of the total mucosal volume, presumably corresponds to the lamina propria (105). In the present studies the lamina propria was between 34 and 46% of the mucosal volume (I, III - V). Thus, judging from these findings, no shrinkage of the epithelial cells occurred during preparation of the tissue in spite of the hypertonic fixative.
The stereological techniques
The stereological techniques and errors connected with them have been discussed in detail in the material and method section and in Paper I. However, it must again be pointed out that since all the morphological data suffer from systematic and personal errors, attention should be paid to comparisons between data from the different groups rather than to the absolute values.

Parital cell regeneration
In the present studies it became clear that parietal cells do regenerate in mucosal wounds. The difference of opinion in this respect might be explained by too short observation times (35) and different sizes of the gastric lesions (41). A contributing factor is also the definition of what is a parietal cell. Tahara (39) identified these cells, electron microscopically, on the third day after wound operations in mice. However, his definition of an immature parietal cell - low density matrix, numerous ribosomes, and an abundance of small light mitochondria - seems inappropriate, since other cell types, e.g. intraepithelial lymphocytes (106) could be taken for being parietal cells using these criteria. For positive identification, intracellular canaliculi should be identified (I).

Using this stringent criterion it was found that parietal cells appeared after 90 days of healing. An indirect confirmation of that the present ulcer model results in standardized wounds was provided by the fact that parietal cells were found in all 90 days old wounds (I - V), and the variation in the different parameters measured in these cells was not larger than in normal parietal cells.

In spite of a long follow up time, 260 days, there was not a complete restitution of the mucosa. It remained thinner than normal, and the density of gastric glands stayed subnormal (I and Fig. 6). Furthermore, the total parietal cell volume never reached normal levels (I and Fig. 6). Therefore, it was concluded that the mucosa in a healed gastric ulcer will not be entirely normal.
In contrast, the size and ultrastructure of the parietal cells found in the 260 days old wounds was completely normal (I). This was, however, no guarantee for an acid secreting capacity since extracellular structures such as nerve terminals and receptors might not regenerate. This would result in parietal cells which were refractory to stimulation. The existence of such "ineffective cells" has been proposed in Zollinger-Ellison patients (107).

The secretory function was investigated in immature parietal cells from 90 days old wounds (II). These cells were chosen since a normal response to stimulation recorded in this group would strongly indicate normal function also in more mature cells. Stimulation by conventional secretagogues (II) resulted in a dramatic increase in the secretory surface area in the immature as well as the normal parietal cells from the same animal. Thus, since regenerating parietal cells responded morphologically to stimulation it must be assumed that both intra- and extracellular factors responsible for the secretion of acid were, at least partly, restituted at this early stage of healing.

The tubulo-vesicles are also known to react on stimulation (70 - 71), but these structures were not measured in this investigation (II) since the secretory surface appears to be a more sensitive indicator of stimulation (72). Moreover, quantitation of the tubulo-vesicular surface density is difficult to carry out with accuracy.

An extensive system of intracellular canaliculi is not necessarily equivalent with secretion of hydrochlorid acid (108), but secretion of strong acid was confirmed by applying small pH-paper chips on different areas of exposed mucosa in anesthetized rats (II). The pH in the wounds turned out to be the same as in normal mucosa beside the lesions both before and after stimulation. Since the pH was very low in these areas, 3 to 4 before and 2 to 3 after stimulation, the acid cannot be organic and local production of hydrochloric acid must therefore be assumed.
It could be claimed that the acid on the wound surface might originate from the normal mucosa beside the wounds. However, the wound margin prevents such a flow, not only mechanically as a slightly elevated ridge demarcating the wound area from the surrounding undamaged mucosa but, perhaps more important in this context is the neutral or alkaline secretion produced by glands in the wound margin (II, 109). This area would thus act as a barrier, and acid from the surroundings would at least to some extent be neutralized.

Effects of physiological and pharmacological stimulation on the parietal cell regeneration

Ever since the days of Pavlov it has been known that stimulation of the vagal nerves is followed by acid secretion (110). In similarity with other stimuli, activation of the vagi will produce typical changes in the parietal cell ultrastructure, and against this background it is conceivable that cholinergic stimulation might exert a trophic influence on the parietal cell mass, just as gastrin does (78). To retain a normal gastric morphology an intact vagal innervation would then be required. If the vagal influence on the stomach is broken by simply cutting these nerves there is a sharp decline in basal acid secretion (111 - 112) and in the acid response to feeding (80).

The results from previous investigations on the effects of vagotomy on normal gastric mucosa differ, with some authors describing a decrease in the parietal cell mass (76, 113) and changes in their ultrastructure (114). Others (74 - 75, 115) have seen transient alterations, and finally there is a group of investigators who have failed to detect any "disuse atrophy" or other alterations in the parietal cell mass after vagotomy (73, 116 - 120).

In the present study (III) no degenerative changes were observed in normal mucosa after truncal vagotomy with pyloroplasty. In contrast, the vagotomy seemed to counter the hypotrophic alterations in the parietal cells which followed pyloroplasty. In this latter group the parietal cells were smaller than in the vagotomy with pyloroplasty
group at 90 days and after 130 days regeneration there was a significant increase in the parietal cell volume density in the vagotomized, normal mucosa compared both to normal, sham operated, controls and to the corresponding pyloroplasty group. Thus, in normal mucosa in rats with pyloroplasty, a vagotomy seemed to produce a "morphologically trophic effect" on the parietal cells. These results are supported by a recent report by Amano (75) who studied the parietal cells in peptic ulcer patients after vagotomy. After an initial drop he found a progressive increase in the number of parietal cells. His results also offer an explanation for the differences in results between previous investigators in that a time factor seems to be of importance.

In the regenerating wounds truncal vagotomy together with pyloroplasty resulted in retarded morphological maturation of the parietal cells. However, the same changes were seen after pyloroplasty alone, and the significance of the vagotomy was therefore uncertain.

Thus, pyloroplasty in itself resulted in hypotrophic changes in the parietal cells, especially if they were immature. This was not surprising since bile reflux, which can be expected after this operation, induces gastritis with changes in the gastric mucosa (121).

Another factor which stimulates the parietal cells to secrete acid is the hormone gastrin. There is general agreement that gastrin, and the synthetic analogue pentagastrin, act as trophic agents in the gastrointestinal mucosa (78), and administration increases DNA-synthesis (78, 122) and mitotic activity (123) in this region. Long-term treatment produces hyperplasia of the parietal cell mass (124) and this is most likely a consequence of a stimulatory effect on the progenitor cell population (125). In contrast, decreased gastrin levels usually result in hypoplasia of these cells (58, 126).

To investigate the effects of this hormone on the parietal cell structure and regeneration two series of experiments were performed, viz.
antrectomy (III), which decreases the serum gastrin levels (77), and long-term administration of pentagastrin (IV), which transiently raises serum gastrin levels (127).

The results demonstrated that normal levels of gastrin were required to retain normal ultrastructure and size of the parietal cells (III) and to obtain optimal conditions for parietal cell regeneration in gastric wounds (III, IV). It was also shown that gastrin levels above normal (IV) did not enhance the maturation of immature parietal cells and the ultrastructure of normal parietal cells also remained unaltered. Instead, the trophic effect was reflected in an increase in the total volume of parietal cells both in healing wounds and in normal mucosa. Thus, the trophic effect seemed to be exerted on the progenitor cells as suggested earlier (125).

H₂-receptor blockade and parietal cell regeneration and morphology
The H₂-receptor antagonist cimetidine is today widely used in the treatment of the peptic ulcer disease, and its effects on acid secretion have been extensively studied (128). There are indications that long-term treatment might result in an increased parietal cell sensitivity to small doses of histamine (129) with a "rebound" or increased acid secretion as a consequence when cimetidine medication is discontinued. Others (130) have not been able to confirm this phenomenon. Against this background it was interesting to investigate whether normal or regenerating parietal cells were influenced morphologically by long-term cimetidine treatment.

It was found that treatment with cimetidine for 130 days (V) resulted in a large increase of the secretory surface area in both normal and immature parietal cells. Since hydrochloric acid is produced at this site (68) it appears that an increased secretory capacity could be expected. Thus, this finding presents an explanation for the increased sensitivity to histamine which has been observed by other investigators (129).
It was not possible to indicate any direct cause for this increase in secretory surface area. At the time of sacrifice the serum gastrin levels were about the same in the cimetidine treated rats as in the controls, which appears to exclude gastrin as the responsible factor.

SUMMARY AND CONCLUSIONS

Using a modified hemostat wounds were cauterized in the rat gastric mucosa. By standardizing the temperature and pressure of the instrument lesions were obtained which were of the same size and depth in all animals. Histologically, they resembled the chronic gastric ulcer in man. The method turned out to be appropriate when quantitation of different cell parameters is performed.

Parietal cells regenerated in these lesions and, after 260 days of healing their ultrastructure was completely normal. However, the volume fraction of these cells, and of gastric epithelium, remained subnormal.

Immature parietal cells, found in 90 days old wounds, responded morphologically to stimulation by the secretagogues pentagastrin and carbacholine, and strong acid was secreted.

Pyloroplasty retarded maturation of the parietal cells, and the morphology of normal cells also changed. If truncal vagotomy was added to the pyloroplasty these latter changes disappeared. It would therefore seem that truncal vagotomy might produce a "trophic effect" on the morphology of normal parietal cells in rats with pyloroplasty.

Normal gastrin levels were needed to obtain optimal conditions for parietal cell maturation and to retain the structure of the parietal cells in normal mucosa. Levels below normal, obtained by antrectomy, resulted in smaller cells with a smaller volume of mitochondria and in a thinner mucosa. Levels above normal, obtained by administration of pentagastrin, did not affect parietal cell structure but the number of
these cells increased. This was probably an effect of stimulation of
the progenitor cell population.

Discontinuation of long-term treatment with the H$_2$-receptor antagon-
ist cimetidine was followed by a large increase in the secretory sur-
face area of both normal and regenerating parietal cells. Since hydro-
chloric acid is produced at the membranes lining these structures, an
increased production of acid must be considered. This finding supports
the view that long-term cimetidine treatment increases the parietal
cell sensitivity to stimulation.

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