REFLUX OESOPHAGITIS IN THE RAT

The damaging action of pancreatic juice

The development of mural fibrosis

PROEFSCHRIFT

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CONTENTS

CHAPTER 1: INTRODUCTION

In the development of symptomatic gastro-oesophageal reflux disease in man the following pathophysiological mechanisms are of importance:

1. increased frequency and prolonged duration of periods of gastro-oesophageal reflux due to lower oesophageal sphincter incompetence (DeMeester et al., 1976);
2. delayed oesophageal clearance of the refluent gastric contents (Little et al., 1980);
3. delayed gastric emptying (Donovan et al., 1977; Little et al., 1980);
4. increased duodenogastric reflux (Kaye and Showalter, 1974; Crumplin et al., 1974; Stol et al., 1974; Donovan et al., 1977).

The net result of these phenomena is a prolonged and repeated exposure of the oesophageal mucosa to potentially damaging substances originating from gastric and duodenal secretions. This leads to pathological changes that can vary from mild epithelial damage with hyperplasia of basal cells, acanthosis and inflammatory cells in the tunica propria (Ismael-Beigi et al., 1970), to panmural oesophagitis with mucosal ulcerations and transmural inflammatory changes (Sandry, 1962).

The natural history of the disease is not well known. A number of patients develop fibrous strictures. The exact incidence of this serious complication is unknown.

Ingram et al. (1960) conducted a histopathologic study of experimental surgical reflux oesophagitis in dogs and concluded that reflux oesophagitis proceeded through a sequential pattern of destruction and healing. They divided the histopathologic characteristics of the disease in four stages: stage 1: epithelial necrosis; stage 2: loss of epithelium with scattered inflammatory cell infiltration in the lamina propria; stage 3: acute ulceration with destruction of the normal architecture of the mucosa alone or together with the submucosa; stage 4: chronic ulceration, which may persist if extensive or may heal completely. Strictures did not develop in the animals used in this study.

Kranendonk (1980) described the sequence of pathological changes in surgically induced reflux oesophagitis in rats and noticed the following events: epithelial erosions and ulcerations intermingled with areas of hyperplasia and hyperkeratosis, a dense inflammatory infiltrate in the submucosa and the muscle wall, and increasing mural fibrosis. Also in this study strictures did not develop. According to Ingram (1960) healing can be part of the natural history of the disease, but in human oesophagitis medical or surgical treatment is usually necessary to interfere with the gastro-oesophageal

reflux after which healing can take place. Whether, as in Ingram's experiment, healing is complete without residual fibrosis in the wall is unknown.

When considering the nature of the offensive agent in the refluent gastric contents, active components from gastric juice as well as from duodenal juice must be taken into account. The two theories that deal with the aetiology of reflux oesophagitis with respect to the offensive agent will be reviewed under the headings of "gastric juice theory" and "duodenal juice theory".

1.1. GASTRIC JUICE THEORY

1.1.1. Clinical studies

Quincke (1879) was the first to draw attention to distal oesophagitis in relation to the digestive action of acid gastric juice. The concept of injury to the oesophageal mucosa by gastric juice was further developed by Hamperl (1934, cited by Postlethwait, 1979), who introduced the term "peptic oesophagitis" as an expression of the supposed damaging potential of gastric acid and pepsin. This theory is further referred to as the "gastric juice theory". Based on this gastric juice theory Wangensteen and Leven (1949) and Casten (1967) performed acid-reducing operations for the treatment of reflux oesophagitis and both reported satisfactory short-term results.

The recently developed method of 24-hour pH monitoring in the distal oesophagus has demonstrated that prolonged and repeated periods of pH below 4 occur in symptomatic gastro-oesophageal reflux patients (DeMeester et al., 1976). The occurrence of these periods of pH below 4 has been shown to correlate well with epithelial changes of the oesophageal mucosa in the sense of increased papillary length and basal cell hyperplasia (Johnson et al., 1978). The results of the 24-hour pH measurements are thought to confirm the theory of gastric juice induced damage to the oesophagus.

1.1.2. Experimental studies

Experiments designed to identify the offensive agent in reflux oesophagitis are either perfusion experiments in which the substance under study is dripped continuously into the oesophagus of an anaesthetized animal, or experiments in which the lower oesophageal sphincter is bypassed or destroyed, so that reflux of gastro-intestinal juices may occur. In this latter type of experiments the presence in the oesophageal contents of the

substance(s) that is (are) thought to regurgitate has never been demonstrated.

Selye (1938) ligated the pylorus of rats and noticed the development of perforating oesophageal lesions within 12 to 18 hours, and ascribed these lesions to the action of refluent gastric juice. Shay (1945) in a similar rat experiment noticed only gastric ulcers, and did not report oesophageal lesions. Arroyave et al. (1950) implanted a functioning gastric mucosal patch in the canine oesophagus and reported the development of ulcers around the patch, which in their view confirmed the gastric juice theory.
Ferguson et al. (1950) perfused the oesophagus of dogs and cats with acid gastric juice which resulted in oesophagitis. Hydrochloric acid alone had only a mild damaging effect, whilst the addition of bile or pancreatic juice abolished the damaging effect of gastric juice. They also repeated Selye's experiment and in contrast to Shay (1945) confirmed Selye's results.
Redo et al. (1959) performed another perfusion experiment in the dog and concluded that unaltered gastric juice was an important factor in the development of reflux oesophagitis provided the pH was below 2.1. The addition of bile made gastric juice less offensive.
Levrat et al. (1962) and Kranendonk (1980) induced reflux of gastric contents into the rat oesophagus surgically, and saw no damaging effect from gastric juice. Goldberg et al. (1969) perfused the feline oesophagus with different acid-pepsin solutions resulting in a maximal damaging effect at a pH of 1.6.
Gillison et al. (1972) demonstrated that surgically induced gastric juice reflux in monkeys after excision of the gastro-oesophageal junction resulted in only a very mild oesophagitis.
Goodale et al. (1980) showed a direct relation between the concentration of pepsin in the perfusate and the perforation rate of the oesophagus in cats.

It has been demonstrated that an increase of H^+-ion transport across the mucosa of the stomach is an early sign of acute gastric mucosal injury (Ritchie, 1977). A similar mechanism is thought operative in oesophageal mucosal injury.
Chung et al. (1974) showed that contact of short duration with high acid concentrations and prolonged contact with lower acid concentrations caused an increase of oesophageal transmucosal H^+-ion transport in rabbits. Histologically this finding correlated with the development of submucosal oedema and ulcerations. These findings were later confirmed by Orlando et al. (1979).
Safaie-Shiraz (1977) showed that pepsin in the presence of acid increased the permeability of canine oesophageal mucosa to H^+-ions.

In conclusion, these experimental studies seem to demonstrate a definite damaging effect of the combination of acid and pepsin on the oesophageal

mucosa in the species tested. However, in all experiments a continuous exposure of the oesophageal mucosa to the substance under study was necessary for such prolonged periods of time, which are not found in the 24-hour pH measurement studies in man. The clinical relevance of these findings therefore remains to be established.
The interpretation of the results of acid reducing operations is hampered by the lack of follow-up studies.
As to the interpretation of 24-hour pH studies it must be kept in mind that a low pH in the distal oesophagus could be an indicator of gastro-oesophageal reflux and not a measure of the damaging factor itself.

1.2. DUODENAL JUICE THEORY

1.2.1. Clinical studies

Oesophagitis due to gastro-oesophageal reflux in the absence of acid and pepsin is a well established clinical entity in patients with achlorhydria (Palmer, 1960), and in patients after a partial (Cox, 1961), or total gastrectomy (Helsingen, 1961). Offensive agents from duodenal juice are thought to be responsible for the development of oesophagitis in these circumstances. The noxious action of duodenal contents in the oesophagus has also been suggested in symptomatic gastro-oesophageal reflux patients with a normal secreting stomach (Gillison et al., 1971; Rovati et al., 1971). Pellegrini et al. (1978) demonstrated periods of alkaline gastro-oesophageal reflux by means of 24-hour pH monitoring in the distal oesophagus.
Duodenal contents that are to reach the oesophagus must pass through the stomach. In symptomatic gastro-oesophageal reflux patients several authors demonstrated concomitant increased duodenogastric reflux (Clémencon, 1972; Kay and Showalter, 1974; Crumplin et al., 1974; Stol et al., 1974; Donovan et al., 1977).
The potentially injurious components of duodenal juice are excreted in bile or pancreatic juice or formed in the duodenal lumen. Bile salts are considered to be the corrosive components of bile. Safaie-Shiraz et al. (1975) have shown that bile salts in the presence of acid cause a five-fold increase in transmucosal transport of H^+-ions in human oesophagi. Gillison et al. (1971) were able to correlate the presence of bile salts in the oesophagus to the symptom of heartburn.
The corrosive components in pancreatic juice are active proteolytic enzymes like trypsin, chymotrypsin and carboxypeptidase (Harper et al., 1979), and

also lipase (Bateson et al., 1981). The proteolytic enzymes are generally thought to be rapidly inactivated in the stomach. However, experiments by Heizer et al. (1965) have demonstrated that inactivation of trypsin by pepsin takes place at a pH below 3.5. In the absence of pepsin, trypsin is stable in acid solutions and present in an active form (Northrop and Kunitz, 1948). Furthermore, Wenger and Trowbridge (1971) demonstrated the presence of active trypsin in the stomach at a pH between 3.5 and 7 for as long as 90 minutes after a testmeal. Active pancreatic proteolytic enzymes must therefore be considered in the duodenal juice theory of reflux oesophagitis. The possible damaging effect of lipase was suggested by Bateson et al., (1981), who incubated human oesophageal mucosal biopsy specimens in solutions containing lipase and noted epithelial damage.
The admixture of bile and pancreatic juice in the duodenum results in the hydrolysis of lecithin (excreted in bile) to lysolecithin under the influence of phospholipase A (excreted in pancreatic juice). The damaging action of lysolecithin on gastric mucosa is well documented (Johnson and McDermott, 1974; Kivilaakso, 1976, 1978). Reports concerning the action of lysolecithin on human oesophageal mucosa are not available.

1.2.2. Experimental studies

Surgically induced duodenal juice reflux into the oesophagus after total gastrectomy has been shown to cause oesophagitis in dogs (Cross and Wangensteen, 1951) and in rats (Helsingen, 1959-1960). Cross and Wangensteen induced separate reflux of bile and pancreatic juice which was followed by oesophagitis in both cases. Perfusion of the feline oesophagus by the same authors resulted in oesophagitis when a mixture of bile and pancreatic juice, bile alone or sodium taurocholate alone was used.
Levrat et al. (1962) studied surgically induced reflux oesophagitis in rats and found bile to be more harmful than pancreatic juice.
Lambert et al. (1962) performed a similar rat experiment and concluded the opposite. They thought that bile could only have a potentiating effect on the corrosive action of pancreatic juice. Lambert (1962) managed to partially prevent oesophageal lesions by the daily intraperitoneal administration of a trypsin inhibitor.
Moffat and Berkas (1965) diverted bile into the canine oesophagus which resulted in erosive oesophagitis.
Gillison et al. (1972) induced reflux of gastric juice mixed with bile in the Rhesus monkey after which oesophagitis developed. Gastric juice reflux without bile resulted in a very mild or no oesophagitis.

Henderson et al. (1972, 1973) perfused the canine oesophagus with bile, bile salts and hydrochloric acid in single solution and in combination. They concluded that bile salts, particularly taurocholate and glycocholate caused oesophagitis provided the presence of hydrochloric acid ensured a pH of 2. Safaie-Shirazi et al. (1975) exposed the canine oesophagus for four and a half hours to a solution of bile salts in acid and this caused oesophageal ulcerations. They also measured a significant loss of H^+-ions from the oesophagus, and concluded that the oesophagitis and ulcerations they found in association with intra-oesophageal bile and HCl might have been due to increased permeability of the oesophageal mucosa to H^+-ions induced by bile salts.

Kranendonk (1980) performed a randomized study in rats which were subjected to surgically induced reflux of different gastro-intestinal juices into the oesophagus. He found that oesophagitis developed only when pancreatic juice was part of the refluent bolus. However, in his experiment he did not actually prove the presence of the refluent juice under study in the oesophageal contents.

Kivilaakso et al. (1980) tested the effect of bile salts and related compounds in an elegant in vitro experiment on rabbit oesophageal mucosa. They measured mucosal potential difference, tissue electrical resistance and the tissue permeability to H^+-ions in acid circumstances. Their data suggest that in the presence of H^+-ions, pepsin and conjugated bile salts are the substances responsible for mucosal injury; however, in the absence of H^+-ions, trypsin and deconjugated bile salts are the crucial factors. Lysolecithin had but a mild damaging effect.

Harmon et al. (1981) have demonstrated in a perfusion experiment in rabbits an increased permeability of the oesophageal mucosa to H^+-ions after exposure to a test solution with a pH of 1. At a pH of 2 the addition of conjugated bile salts caused an increase in the permeability to H^+-ions, which varied directly with the concentration of the bile acids and the duration of the exposure. Unconjugated bile salts were shown to increase the permeability to H^+-ions at a pH of 7.

In conclusion, these experimental data seem to demonstrate that components from bile and pancreatic juice can cause oesophageal damage under the conditions of the experiments and in the species tested. The clinical data are consistent with a duodenal origin of the damaging factor(s) in reflux oesophagitis.

1.3. GENERAL COMMENT

The experimental designs of perfusion studies can be criticized for the unphysiological conditions that are created when the oesophagus of an anaesthestized animal is exposed to a test solution for a prolonged period of time. The majority of the experiments performed with surgically induced reflux are not randomized, are devoid of control groups, but certainly all lack control data concerning the oesophageal contents, proving the actual occurrence of the supposedly induced reflux.

1.4. MOTIVATION FOR THE PRESENT STUDY

The results obtained in a recent study of surgically induced reflux oesophagitis in rats (Kranendonk, 1980) prompted the study presented in this thesis. In the randomized study by Kranendonk (1980) the reflux of pancreatic juice invariably resulted in oesophagitis; admixture of bile and/or gastric juice caused no significant differences in the extent of the oesophagitis. The reflux of bile or gastric juice was not associated with the development of oesophageal lesions. However, the study lacked control data on the composition of oesophageal contents as proof of the supposed reflux. Furthermore Kranendonk (1980) described the development of mural fibrosis starting after 14 days of reflux and progressing with time. The concomitant inflammatory changes, however, made a good interpretation of the presence of fibrosis impossible. It was therefore decided to further study surgically induced reflux oesophagitis in rats in order to prove the supposed reflux and to further study mural fibrosis.

Being aware of the anatomical differences between the oesophagus of rats and of the human being, the rat was none the less chosen as experimental animal because of the ready availability of a good experimental model. In addition, rats can be kept in relatively large numbers, allowing more elaborated experiments with appropriate statistical analysis.

1.5. OBJECTIVES OF THE PRESENT STUDY

1. To determine whether active pepsin can be found in the oesophagus of rats after a gastric juice reflux inducing operation, and whether the presence of pepsin could be related to the development of oesophagitis.

2. To study the vagal innervation of the rat stomach after oesophago-jejunostomy.

3. To study in surgically induced reflux in the rat, whether trypsin and bile acids are present in oesophageal sample before and after the reflux inducing operation, and whether their presence correlates with the occurrence and degree of oesophagitis.

4. To study the relation between the duration of reflux and the biochemically determinated collagen content of the oesophageal wall in surgically induced reflux oesophagitis.

5. To study the consequences of healing of surgically induced reflux oesophagitis in rats after a reflux abolishing operation in relation to the duration of reflux and at different time intervals after the reflux abolishing operation.

CHAPTER 2: MATERIALS AND METHODS

2.1. EXPERIMENTAL ANIMALS

In all experiments male adult Wistar rats were used. In the experiments described in Chapters 3 and 4 their mean age was 20 weeks with weights ranging from 250 to 350 g. In the experiment described in Chapter 5 their mean age was 15 weeks and their weight ranged from 200 to 300 g.

2.2. PRE-OPERATIVE NURSING CARE

A maximum of 5 rats were nursed together on fine woodchips in a perspex box with a wire roof. They were fed commercial rat chow (Hope Farm B.V., Woerden) and drank tap water that was acidified with hydrochloric acid to a pH of 3.3 to prevent bacterial growth.

2.3. METHOD OF RANDOMIZATION

Randomization was accomplished by allotting numbers to each animal in the planned experimental and control groups. The numbers were written on pieces of cardboard and put in a box. Before an animal was operated upon a number was drawn and the indicated procedure performed. In case of premature death of the animal the corresponding number was returned to the box so that it could be drawn again.

2.4. ANAESTHESIA

The rats had free access to food and water until the induction of anaesthesia. Anaesthesia was induced and maintained with an aether-air mixture. Once anaesthetized the rats were weighed and shaved.

2.5. SURGICAL PROCEDURE (GENERAL ASPECTS)

Operations were performed under clean but not sterile conditions. The area for incision was cleaned with an aqueous hexachlorophene solution. The abdomen was opened through a midline incision extending from xyphoid to pubis. The cardia was exposed by dividing the avascular adhesions between liver and stomach. The left gastric artery and right vagus trunk were separated from the intra-abdominal oesophagus (length $\pm$ 1 cm) and in case

of a total gastrectomy ligated with 3-0 silk. The left vagus was also dissected free from the oesophagus, to make transection of the oesophagus possible without dividing the vagal nerves. The cardia, the duodenal stump in case of a total gastrectomy, and the afferent jejunal limb in case of a reflux-abolishing operation, were ligated with 2-0 silk. During a total gastrectomy all gastric vessels other than the left gastric artery were electrocoagulated with a bipolar dissecting forceps. All bowel anastomoses were performed in one layer with atraumatic 7-0 silk as a running suture (for technique, see Fig. 2.1). The suture comprised the full thickness bowel wall except in the case of anastomosis with the oesophagus when only the oesophageal mucosa was included in the suture. For anastomosis of the common bile duct to the bowel atraumatic 8-0 nylon was used (for technique, see Fig. 2.2). The abdomen was closed in one layer with a running 2-0 silk suture.

2.6. OESOPHAGEAL WASHOUT TECHNIQUE

Through a median laparotomy, the intra-abdominal oesophagus was occluded with a Bulldog clamp. The oesophagus was then intubated through the mouth using a 1.6 mm Ø polyethylene catheter. A midline cervical incision was used to expose the proximal oesophagus, which was encircled with a vessel loop to be able to occlude the lumen around the catheter. Next the oesophagus was rinsed 5 times with 0.7 ml of normal saline. The sample thus obtained was temporarily stored on crushed ice. After centrifuging for 5 minutes at 3000 rpm the supernatant was frozen to -20° C for further biochemical analysis. Depending on the type of experiment, determinations of trypsin activity, pepsin activity or total bile acids were done. The cervical incision was closed with a running 2-0 silk suture.

2.7. BLOOD SAMPLES

Blood samples were taken by retro-orbital puncture under anaesthesia using heparinized glass capillaries.

2.8. POST-OPERATIVE NURSING CARE

The rats which were subjected to a reflux abolishing operation received 12-15 ml "Ringers" lactate subcutaneously directly after the operation. All rats recovered for about one hour under an infrared lamp. Thereafter they had free access to acidified tap water and rat chow and were nursed on paper shreds to avoid choking due to swallowed woodchips. The animals were weighed once a week.

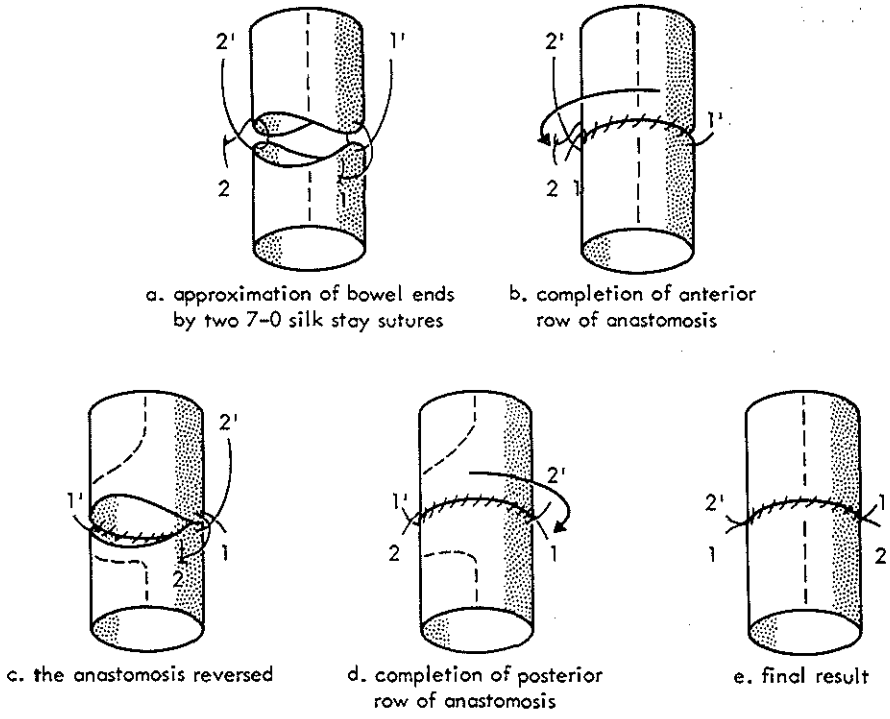

Fig. 2.1. Technique of bowel anastomosis.

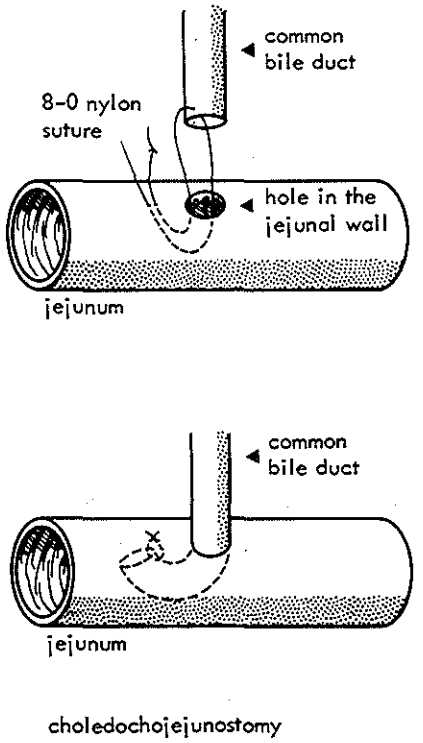

Fig. 2.2. Technique of end-to-side choledochojejunostomy.

2.9. SACRIFICE

At the end of all experiments a second oesophageal washout was done under general anaesthesia. The rats were then exsanguinated by transection of the intra-abdominal aorta. The oesophagus was dissected free from the larynx to the oesophagojejunal anastomosis and removed, including that anastomosis.

2.10. MACROSCOPIC EXAMINATION

The oesophagus was opened longitudinally along its anterior border and pinned to a piece of cork. The length of the oesophagus and the width of the anastomosis were measured. Then the mucosal surface of the oesophagus was examined. The mucosa was considered abnormal in case of redness, (partial) absence of epithelium or hyperkeratosis. The area of abnormal mucosa was quantified with the following formula:

$$\frac{\text{abnormal mucosal surface } (mm^2)}{\text{total mucosal surface } (mm^2)} \times 15 \ *$$

the result of which was called the macroscopic score.

2.11. OESOPHAGEAL WALL SAMPLE

In the experiment on healing of oesophagitis described in Chapter 5, a fullthickness sample of the oesophageal wall was used for collagen content determination. The sample was taken 1 cm proximal to the oesophagojejunal anastomosis and comprised half of the oesophageal circumference (see Fig. 2.3). The tissue was weighed immediately and frozen at -20° C.

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* This formula is identical to that used by Kranendonk in his thesis (1980) The multiplication factor 15 was useful in Kranendonk's study, and is used here to be able to compare the findings in this study with the findings of Kranendonk.

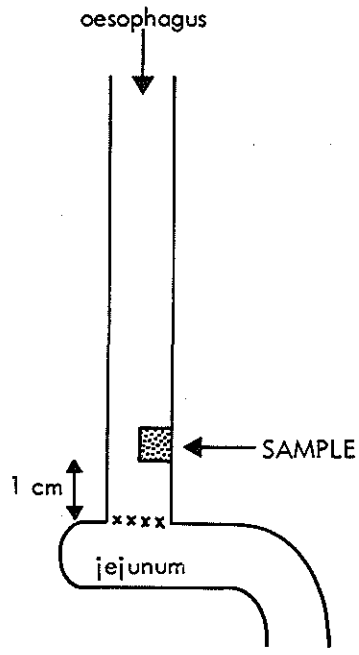


Fig. 2.3. Sampling procedure of the oesophageal wall for collagen content determination. The oesophagus is opened longitudinally and pinned to a piece of cork. At the indicated place a full thickness tissue sample of 5 mm oesophageal length and half the circumference is taken.

2.12. MICROSCOPIC EXAMINATION

2.12.1. Fixing and staining

The total oesophagus was fixed in 10 percent formalin and embedded in paraffin. Longitudinal sections were cut and routinely stained with haematoxylin, azophloxin and saffrane (HAS). On a limited number of sections a specific collagen stain was done with azocarmine and anilin (AZAN).

2.12.2. Definitions

Erosion: a complete or partial destruction of the epithelial layer without destruction of the tunica propria or deeper structures;
ulceration: complete destruction of the epithelial layer and damage to the tunica propria or deeper structures;
basal cell hyperplasia: more than two layers of basal cells.

To be able to define three other abnormal findings, namely hyperkeratosis, hyperplasia of the epithelial layer and hyperplasia of the muscularis mucosae we first obtained normal values for the thickness of these layers. The oesophagi of five non-operated rats were each measured in five different places with an ocular measure at a magnification of 400x. The following mean values and standard deviations were obtained, expressed in micrometers:

keratin layer	27.0	SD 19.8
epithelium	38.4	SD 16.8
musc. mucosae	16.8	SD 7.2

Maximum values of normal thickness were defined as $\bar{x}$ + 2 SD.

2.12.3. Criteria for oesophagitis

Oesophagitis was diagnosed if one or more of the following were found:
- erosive or ulcerative epithelial defects;
- polymorphonuclear infiltration of the oesophageal wall;
- hyperplasia of the epithelium;
- hyperkeratosis.

These criteria for the diagnosis of oesophagitis are based upon histological studies of reflux oesophagitis in rats by Levrat et al. (1961) and Kranendonk (1980). In addition to the histological phenomena described above we also employed the criteria that were described by Ismael-Beigi et al. (1970):
- hyperplasia of the basal cell layer;
- elongated lamina propria papillae.

2.13. STATISTICAL ANALYSIS

Paired data were analyzed with Wilcoxon's signed rank test, and unpaired data with Wilcoxon's rank sum test.

The relation between different variables in individual rats was investigated with Spearman's rank correlation test.

In case of combination of the results of different experimental groups or control groups, respectively, to form one larger category the Kruskal and Wallis test was employed, in order to study whether the results in the different groups could be considered as belonging to the same population of observations.

All significance tests were carried out with respect to a significance level $\alpha = 0.05$.

CHAPTER 3: ACID, ACTIVE PEPSIN AND REFLUX OESOPHAGITIS

3.1. INTRODUCTION

Reflux of acid and pepsin into the oesophagus has been proposed as aetiological factor in the development of reflux oesophagitis in man (Quincke, 1879). Efforts to support this theory with evidence from animal experimentation have led to conflicting results (see section 1.1.2.).

In rats, Selye (1938) produced ulcers of the supra-diaphragmatic oesophagus after ligation of the pylorus and ascribed them to peptic digestion. He saw no ulcers in the stomach or intra-abdominal oesophagus. However, Shay (1945), after ligation of the pylorus, saw mainly stomach ulcers and did not mention oesophagitis. Lodge (1955) used the method of partial starvation to produce ulcers in both stomach and oesophagus, and concluded from her experiment that the ulcers were due to the action of gastric juice. Levrat (1962) and Kranendonk (1980) surgically induced reflux of gastric juice into the rat oesophagus. In their experiments the intra-abdominal oesophagus was transected at the cardia and anastomosed to the glandular stomach (Levrat, 1962, and Kranendonk, 1980) or to the first jejunal loop (Levrat, 1962) from which duodenal juice had been diverted. Neither of these authors saw oesophageal lesions after these reflux-inducing operations and concluded that, in rats, reflux of gastric juice is not associated with the development of reflux oesophagitis. However, they did not test the composition of the juice that was regurgitating into the oesophagus.

The digestive action of gastric juice is determined by the presence of acid and active pepsin. Acid is necessary for the conversion of pepsinogen into pepsin (Harper et al., 1979). The proteolytic activity of pepsin is dependent on the presence of H^+-ions. The pH optimum for the proteolytic activity of pepsin varies with the substrate (Northrop and Kunitz, 1948), but is thought to be between 1.0 and 2.0 (Harper et al., 1979). Thus the divergent conclusions of the different authors regarding the effect of gastric juice reflux on the rat oesophagus could be due to differences in the proteolytic activity and acidity of the gastric juice of which they were studying the effect.

It is postulated that even when great care is taken to spare the vagal trunks, the transection of the intra-abdominal oesophagus in rats and its implantation in the pre-pyloric stomach or the jejunum is associated with loss of vagal activity. The result is a near-total absence of acid and active pepsin in the gastric juice of these rats which reduces its

proteolytic activity to (almost) nil.

The objectives of this investigation were to study the presence of active pepsin in the rat oesophagus after a gastric juice reflux inducing operation, and to study the vagal innervation of the stomach after oesophagojejunostomy using the Hollander test (1946).

3.2. GASTRIC JUICE REFLUX

3.2.1. Materials and methods

3.2.1.1. Surgical procedure

Ten male adult Wistar rats were subjected to an oesophageal washout procedure as described in section 2.6.

Thereafter gastric juice reflux was induced by the following operation: ligation of the cardia; transection of the intra-abdominal oesophagus carefully sparing the vagal trunks; end-to-side oesophagojejunostomy; ligation of the proximal duodenum; transection of the pylorus; transection of the afferent limb of the first jejunal loop; end-to-end gastrojejunostomy (afferent limb); end-to-side duodenojejunostomy 25 cm distally to the oesophagojejunostomy (Fig. 4.3).

3.2.1.2. Autopsy

One week after the reflux inducing operation the rats were anaesthetized and the oesophageal washout procedure was repeated. The stomach was openend and the pH of its content determined with a paper strip (Lyphan pH paper). The rats were killed under anaesthesia and the oesophagus was removed for macroscopic and microscopic examination.

3.2.1.3. Biochemical analysis

The oesophageal washout samples were analysed for pepsin activity and trypsin activity. Pepsin activity was determined using a hemoglobin digestion method (Rick and Fritsch, 1970). Trypsin activity was determined with a kinetic method using benzyl-phenylalanyl-L-val-L-arginyl-p-nitroanilide (S-2160, Kabi Vitrum B.V., Amsterdam) as a substrate (Bergström, 1977).

3.2.2. Results

3.2.2.1. General

Ten rats were operated upon with one postoperative death due to a technical failure.

3.2.2.2. Oesophagitis

None of the rats had either macroscopic or microscopic signs of oesophagitis.

3.2.2.3. Active pepsin determinations

In none of the oesophageal washout samples obtained either pre- or postoperatively any pepsin activity could be demonstrated.

3.2.2.4. Active trypsin determinations

In all pre- and postoperative samples a small amount of active trypsin was found. There was no significant difference between pre- and postoperative samples.

3.2.2.5. pH of gastric contents

In all rats the pH of gastric contents was found to be between 5 and 6.

3.3. HOLLANDER TEST

3.3.1. Introduction

It was shown by Lin and Alphin (1958) that the main pathway for the cephalic phase of gastric secretion in the rat is the vagus. The cephalic phase of gastric secretion can be stimulated by insulin-induced hypoglycaemia (Hollander, 1946) provided the vagus is intact. Hypoglycaemia is adequate in stimulating gastric secretion when blood glucose levels are below 2.7 mmol/l (Hollander, 1946). The optimum dosage of insulin in rats is 4 U of crystalline zinc insulin per 100 gram rat body weight (Lee and Thompson, 1967).

3.3.2. Materials and methods

3.3.2.1. Surgical procedure

Adult male Wistar rats were used. Oesophageal washout was not performed. The intra-abdominal oesophagus was dissected free carefully sparing the vagal nerves. The cardia was ligated and the intra-abdominal oesophagus transected; the oesophagus was then anastomosed to the first jejunal loop (end-to-side oesophagojejunostomy, Fig. 4.6.).

3.3.2.2. Test for vagal innervation (Hollander, 1946)

The rats were fasted overnight. At zero hour they were given a subcutaneous injection of crystalline zinc insulin, dosage 4 Units per 100 gram rat (Lee and Thompson, 1967) or a similar amount of normal saline, after being anaesthetized. A midline laparotomy was performed and the pylorus ligated. The abdominal incision was closed and anaesthesia ended. Blood glucose levels were determined at 0, 1 and 2 hours (Ames Dextrometer). Two hours after insulin injection laparotomy was repeated and gastric contents were aspirated. The pH of these contents was established with a pH electrode (Radiometer pH 26, Copenhagen).

The diagnostic value of the test was determined in 13 non-operated rats of whom 8 were injected with insulin and 5 with an equal amount of normal saline, thus serving as a control group. The rats that were subjected to oesophagojejunostomy were tested fourteen days after this operation. Five received an insulin injection and five a saline injection.
At the end of the test the rats were exsanguinated and the oesophagus of the operated rats removed for macroscopic examination.

3.3.3. Results

3.3.3.1. General

Twelve rats were subjected to an oesophagojejunostomy of which 2 died due to technical failures.

3.3.3.2. Oesophagitis

Macroscopic evidence of oesophagitis was found in all rats that had

been subjected to oesophagojejunostomy.

3.3.3.3. Bloodglucose levels

In all rats that received insulin injection adequate hypoglycaemia was induced (according to Hollander, 1946, bloodglucose levels should be lower than 50 mg% = 2.78 mmol/l).

3.3.3.4. Intragastric pH

In non-operated rats which received insulin injection a mean gastric pH of 1.9 was reached at the end of the test (table 3.I.).

In the operated rats which received insulin injection a mean gastric pH of 5.0 was found.

Saline injection failed to produce hypoglycaemia in either operated or non-operated rats. The average intragastric pH in the non-operated group after saline injection was 6.7, and in the group subjected to oesophagojejunostomy 4.9.

Table 3.I. RESULTS OF HOLLANDER TEST

Group	n	Mean bloodglucose levels in mmol/l (hours after injection)			pH
		0	1	2	
Non-operated, insulin	8	6.1	2.2	1.2	1.9
Non-operated, saline	5	4.7	6.0	4.9	6.7
Operated, insulin	5	5.2	2.5	2.0	5
Operated, saline	5	6.6	10.2	11.7	4.9

The difference in pH between the non-operated groups injected with insulin or saline is statistically significant ($p < 0.01$, Wilcoxon, rank sum test). There is no statistically significant difference between the operated groups injected with insulin or saline, regarding the intragastric pH.

3.4. DISCUSSION

Experiments designed to study the aetiology of reflux oesophagitis have been performed in several animal species (for a review, see sections 1.1.2. and 1.2.2.). Two types of experiments can be distinguished.
Firstly, there are experiments in which the substance under study is continuously dripped into the oesophagus of an experimental animal.
Secondly, there are experiments in which by means of an operation the lower oesophageal sphincter is either destroyed or bypassed in order to allow reflux of certain gastro-intestinal juices. In the description of this second type of experiment, data concerning the composition of the juices that are thought to reflux are usually absent. Therefore, conclusions drawn from these experiments are subject to serious doubts. The results of the present study justify these doubts. Previous authors (Levrat, 1962; Kranendonk, 1980) concluded after similar experiments that in rats reflux of gastric juice into the oesophagus is not associated with the development of oesophageal lesions. The absence of oesophageal lesions was confirmed in this study. However, analysis of the contents of the oesophagus obtained by a washout technique did not demonstrate the presence of active pepsin. Also insulin-induced hypoglycaemia did not result in a gastric acid output that was sufficient to lower the pH to a level at which pepsin is active.

It is therefore concluded that, although theoretically reflux of gastric juice occurs in the model described, the refluent does not contain acid or active pepsin due to an inadvertent, but unavoidable, vagotomy. Thus this model is not suitable to investigate the action of the acid-pepsin complex on the rat oesophagus.

In future experiments designed to study reflux oesophagitis we recommend that the contents of the oesophagus should be analyzed for the presence and activity of the substances that are thought to regurgitate.

3.5. SUMMARY AND CONCLUSIONS

Surgically induced reflux of gastric juice into the oesophagus of rats after a procedure which includes transection of the oesophagus and end-to-side oesophagojejunostomy does not result in oesophagitis. It is postulated that the reflux-inducing operation itself is damaging to the vagus nerve.

An experiment is described in which pepsin activity was measured in the oesophagus after an operation which was intended to induce reflux of gastric juice. We could not demonstrate any pepsin activity. The intragastric pH

after this operation was between 5 and 6.

We also described an experiment in which we tested vagal function in rats, which had been subjected to oesophagojejunostomy, using insulin-induced hypoglycaemia (Hollander test). The results of the Hollander test indicate that after an oesophagojejunostomy it is not possible to stimulate vagally mediated gastric acid secretion.

Summarizing, the results of both experiments clearly show that after an operation intended to induce reflux of gastric juice as done in our study, there is no appreciable secretion of gastric acid and thus no pepsin activity, and that this is most probably due to damage to the vagus nerve.

CHAPTER 4: ACTIVE TRYPSIN, BILE ACIDS AND REFLUX OESOPHAGITIS

4.1. INTRODUCTION

Reflux of duodenal contents has been proposed as a causal factor in the development of reflux oesophagitis in man (Helsingen, 1961; Himal, 1977; Pellegrini, 1978). There is, however, no common opinion as to the nature of the offending agent in duodenal juice.

In rats reflux oesophagitis due to duodenal contents was first demonstrated by Helsingen (1959-1960, 1960), who performed total gastrectomy and investigated the influence of different types of reconstruction of the bowel continuity on the development of oesophagitis. The construction of an end-to-side oesophagojejunostomy to the first jejunal loop invariably resulted in oesophagitis. Gastric juice, bile and pancreatic juice can be made to drain separately or in combination into the afferent jejunal limb and so their effect on oesophageal mucosa can be studied. Such an experiment was carried out by Levrat (1962), who saw oesophagitis only after the induction of reflux of duodenal contents into the oesophagus. He considered bile more harmful than pancreatic juice. Lambert (1962) studied the separate effects of biliary and pancreatic secretions on the oesophagus and concluded that pancreatic secretions were more harmful than bile, though bile had a potentiating effect. He was also able to partially prevent the consequences of reflux of pancreatic juice into the oesophagus by daily intraperitoneal administration of a trypsin inhibitor. Kranendonk (1980) performed a similar experiment, and also concluded that pancreatic juice reflux into the rat oesophagus was associated with a 100% incidence of oesophagitis whilst in the case of bile reflux no oesophagitis was found.

Kranendonk (1980) demonstrated the existence of reflux per se in this model by means of X-ray studies, but neither he nor the other authors quoted have actually shown the presence of duodenal contents in the rat oesophagus in those cases where oesophagitis occurred. The total bile acid concentration can be used as a marker for bile, and active trypsin as a marker for pancreatic juice, and both can be measured in oesophageal contents.

The objective of this investigation was to study, in comparable rat experiments, whether active trypsin and total bile acids are present in oesophageal washout samples before and after a reflux-inducing operation, and whether their presence correlates with the occurrence and degree of oesophagitis.

4.2. MATERIALS AND METHODS

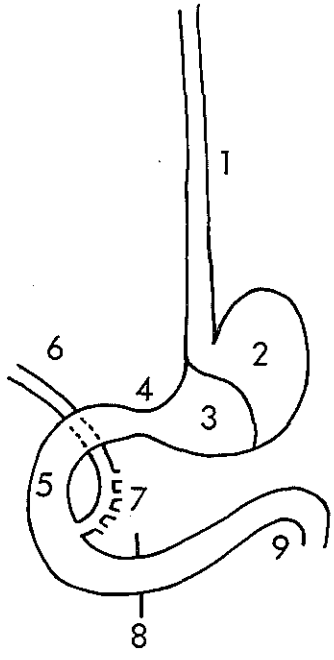
4.2.1. General

Male adult Wistar rats were used in 10 different groups. Of these, 7 were experimental groups made up by the 7 possible combinations of reflux of gastric juice, bile, and pancreatic juice. Three groups served as control groups. A random distribution of the rats as well as a random order of operations was achieved by the method described in section 2.3.

Prior to the selected surgical procedure oesophageal washout was performed in all animals according to the method described in section 2.6.

4.2.2. Surgical procedures

For clarification of the drawings of the anatomical results of the operations a scheme of the normal anatomy of the upper gastro-intestinal tract of the rat is shown in Fig. 4.1.



Scheme normal anatomy

1. oesophagus
2. rumen
3. glandular stomach
4. pylorus
5. duodenum
6. common bile duct
7. pancreatic ducts
8. Treitz' ligament
9. first jejunal loop

Fig. 4.1. Scheme of the normal anatomy of the upper gastro-intestinal tract in the rat.

Control groups: There were three control groups. In the first control group only a laparotomy and an oesophageal washout were performed (n = 10). In the second control group additionally the oesophagus was transected and resutured carefully, sparing the vagus nerves (n = 7). In the third control group an end-to-end oesophagojejunostomy was combined with an end-to-side duodenojejunostomy at 25 cm distance: a Roux-en-Y reconstruction (n = 9), see Fig. 4.2.

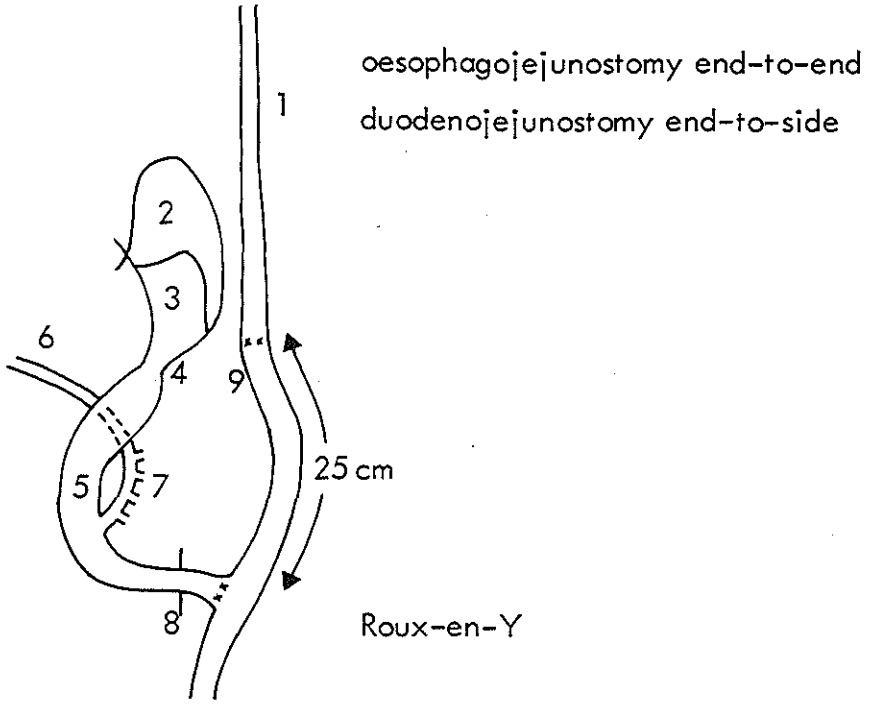
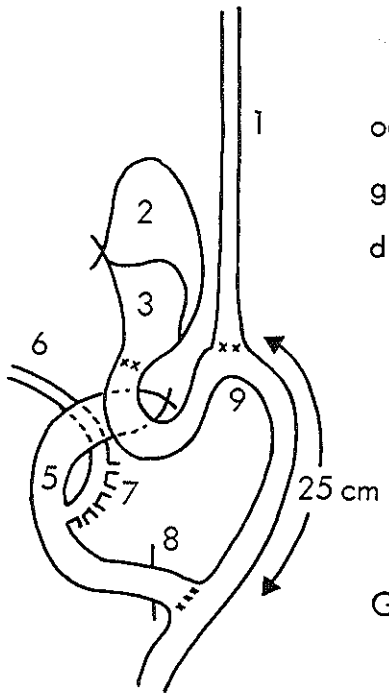


Fig. 4.2. Schematic drawing of the Roux-en-Y procedure.
The figures correspond to those in Fig. 4.1.

Experimental groups: The basic procedure in the experimental groups was an end-to-side oesophagojejunostomy to the first jejunal loop to which either a gastrectomy and/or a deviation of pancreatic or biliary secretions could be added to achieve the required composition of the refluent juice (Table 4.I and Figs. 4.3-4.9).

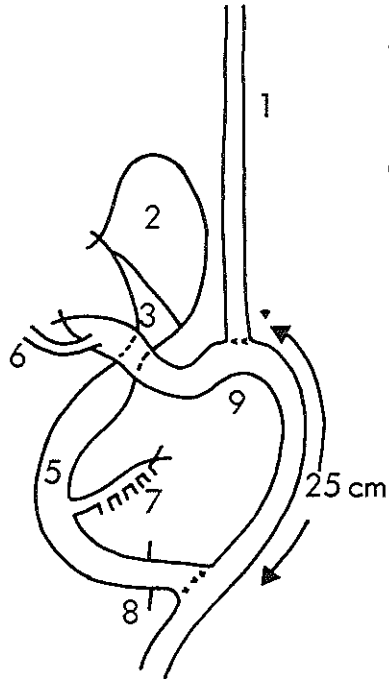
Table 4.I. OPERATIVE PROCEDURES IN EXPERIMENTAL GROUPS

Procedure	Type of reflux induced (G = gastric juice; B = bile, P = pancreatic juice)						
	G	B	GB	GBP	GP	BP	P
ETS oesophagojejunostomy	×	×	×	×	×	×	×
Total gastrectomy						×	×
Gastrojejunostomy (aff. limb)	×		×				
Duodenojejunostomy (eff. limb)	×	×	×				
Choledochojejunostomy (aff.limb)		×	×				
Choledochojejunostomy (eff.limb)					×		×
Number of rats per group	8	9	9	9	8	10	9
Figure	4.3	4.4	4.5	4.6	4.7	4.8	4.9



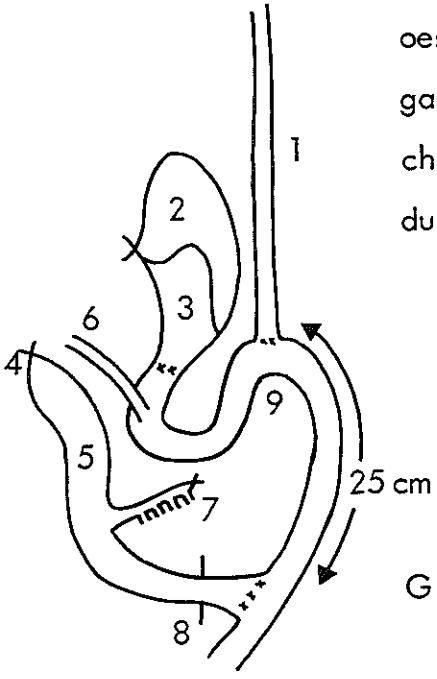
oesophagojejunostomy end-to-side
gastrojejunostomy end-to-end
duodenojejunostomy end-to-side

Fig. 4.3. Operative induction of gastric juice reflux.



oesophagojejunostomy end-to-side
choledochojejunostomy end-to-side
duodenojejunostomy end-to-side

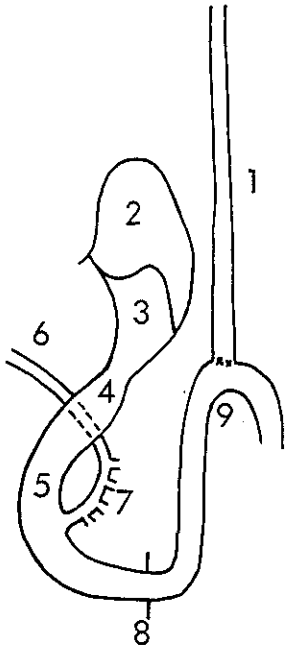
Fig. 4.4. Operative induction of bile reflux.



oesophagojejunostomy end-to-side
gastrojejunostomy end-to-end
choledochojejunostomy end-to-side
duodenojejunostomy end-to-side

G + B

Fig. 4.5. Operative induction of reflux of gastric juice and bile.



oesophagojejunostomy end-to-side

G + B + P

Fig. 4.6. Operative induction of reflux of gastric juice, bile, and pancreatic juice.

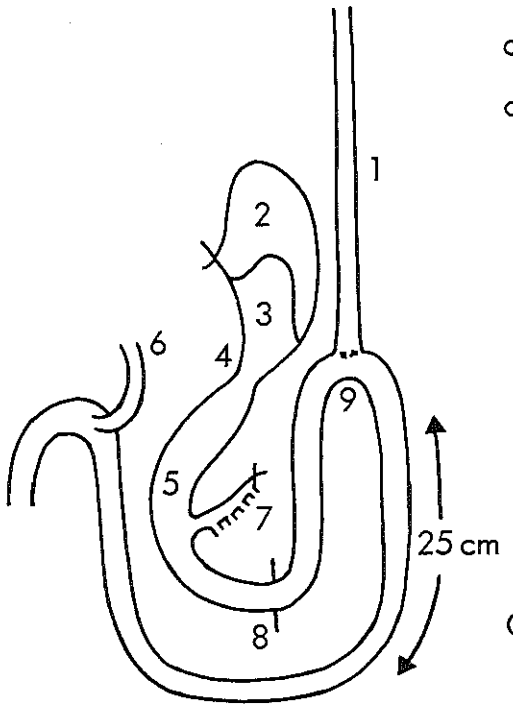


Fig. 4.7. Operative induction of reflux of gastric juice and pancreatic juice.

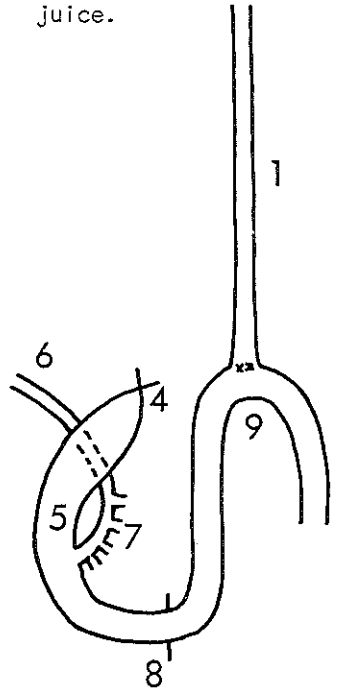


Fig. 4.8. Operative induction of reflux of bile and pancreatic juice.

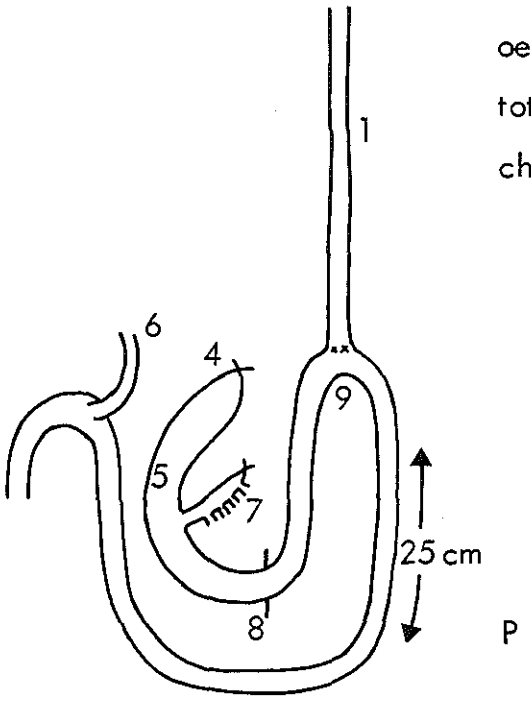


Fig. 4.9. Operative induction of pancreatic juice reflux.

4.2.3. Autopsy

Seven days after the first procedure a second laparotomy was performed. The animals were weighed after being anaesthetized. Again oesophageal washout was done as described. The rats were then exsanguinated and the oesophagus, including the oesophagojejunostomy, was dissected out for macroscopic examination and histopathology studies as described in sections 2.10 and 2.12.

4.2.4. Biochemical analysis

Oesophageal washout samples (1 = preoperative, and 2 = post-operative) were analysed for the presence of active trypsin and bile acids. Trypsin was determined with a kinetic method using benzyl-L-phenylalanyl-L-val-arginyl-p-nitroanilide (S-2160, Kabi Vitrum B.V., Amsterdam) as a substrate (Bergström, 1977). Bile acids were determined fluorimetrically using 3-alpha-OH-steroid dehydrogenase (Mashige, 1976).

4.3. RESULTS

4.3.1. General

A total number of 110 rats were operated upon with a mortality rate of 20% due to technical failures, so that 88 rats could be included in the present study.

4.3.2. Oesophagitis

Macroscopically detected oesophagitis could always be confirmed by histopathology. No histopathologic abnormalities were found when the oesophagus was macroscopically normal.

Oesophagitis was found in all rats in which pancreatic juice had been diverted into the afferent jejunal limb of the oesophagojejunostomy. None of the other animals of the control or experimental groups had signs of oesophagitis (Table 4.II.). This table also indicates the mean weight loss percentage per group one week after operation, showing no significant difference between experimental groups of animals with or without oesophagitis (Kruskal and Wallis test). Three main categories of rats were considered; firstly the rats of the three control groups, secondly the rats in the three experimental groups that did not develop oesophagitis, and thirdly the rats in the four experimental groups that did develop oesophagitis. The Kruskal and Wallis test was used to investigate whether significant differences occurred between groups within each of these three main categories on the basis of comparison of trypsin levels in the post-operative washout samples, and macroscopic scores. There were no significant differences. Therefore, it was justified to statistically compare the results of trypsin and bile acid determinations of these three categories.

Table 4.II. PERCENTAGE OF OESOPHAGITIS AND WEIGHTLOSS

Group	n	% of animals with oesophagitis	mean weight loss %	range
Laparotomy	10	-	3.6	1.2- 6.0
Transection	7	-	15.7	10.3-30.7
Roux-en-Y	9	-	11.0	8.0-18.4
G	8	-	11.5	7.0-20.8
B	9	-	16.0	6.8-26.7
G + B	9	-	14.8	10.0-22.0
G + B + P	9	100	16.3	11.5-19.7
G + P	8	100	16.4	10.0-21.1
B + P	10	100	14.3	10.0-20.4
P	9	100	17.0	12.0-25.6

4.3.3. Trypsin determinations

In all pre-operative washout samples of both control and experimental groups a small amount of trypsin could be demonstrated. In all control and experimental groups the trypsin levels in the post-operative samples were systematically higher than those obtained pre-operatively. Wilcoxon's signed rank test for paired data demonstrated significant differences in all three main categories (see Table 4.III).

Comparison of the post-operative trypsin levels of control groups and experimental groups without oesophagitis showed no significant difference. The difference, however, between the oesophagitis category and both the experimental category without oesophagitis and the control category was statistically significant ($p < 0.01$ in either case), see Fig. 4.10.

Table 4.III. TRYPSIN VALUES IN U/l

CONTROL GROUPS:

Procedure	n	sample 1 med	sample 1 range	sample 2 med	sample 2 range	sample 2 vs sample 1 (Wilcoxon's signed rank test)
Laparotomy	10	5	2-22	10	3-114	
Transection	7	5	2-22	9	2- 68	
Roux-en-Y	9	3	1-13	9	2- 30	
Total	26	5	1-22	9	2-114	$p < 0.01$

EXPERIMENTAL GROUPS:

No oesophagitis

Type of reflux	n	sample 1 med	sample 1 range	sample 2 med	sample 2 range	sample 2 vs sample 1 (Wilcoxon's signed rank test)
G	8	5	4-12	11	4- 38	
B	9	8	1-13	6	0- 53	
B + G	9	5	2-29	6	3- 32	
Total	26	6	1-29	10	0- 53	$p < 0.05$

Oesophagitis

Type of reflux	n	sample 1 med	sample 1 range	sample 2 med	sample 2 range	sample 2 vs sample 1 (Wilcoxon's signed rank test)
G + B + P	9	5	0-29	204	4- 2448	
G + P	8	7	0-10	565	6- 2058	
B + P	10	5	1-12	147	28-11820	
P	9	5	2-12	440	40- 7542	
Total	36	5	0-29	220	4-11820	$p < 0.01$

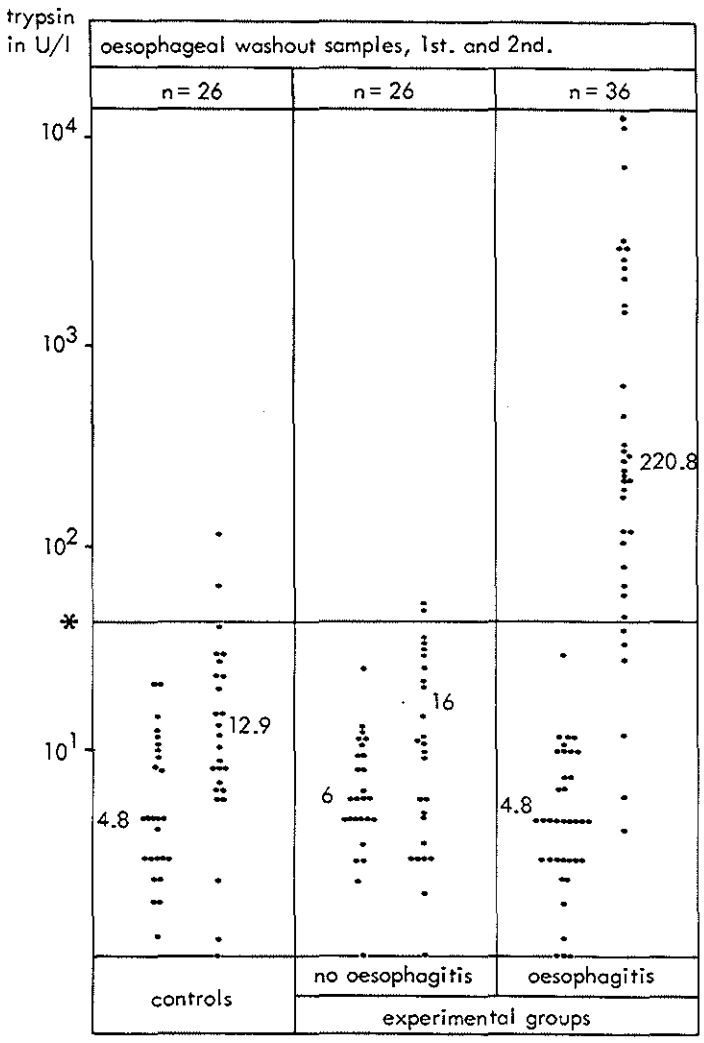


Fig. 4.10. Trypsin concentrations in Units per liter in the three main categories. For each category both pre- (1st) and post-operative (2nd) trypsin levels are shown, using a logarithmic scale on the ordinate. Median values are indicated with each column.
At the asterix the discrimination limit for a positive trypsin test is indicated.

4.3.4. Determination of discrimination limit with respect to trypsin value

Post-operative changes in trypsin concentrations (U/l) were calculated for individual animals from values obtained in pre-operative and post-operative oesophageal washout samples. The 3 control groups and the 3 experimental groups that did not develop oesophagitis, showed very similar results with respect to these post-operative changes (cf. Fig. 4.10); therefore their frequency distribution was obtained, as given below.

Change trypsin value (U/l)	Frequency
-25	1
-15	2
- 5	13
+ 5	18
+15	5
+25	7
+35	2
+45	3
.	.
.	.
+105	1
	52

In view of the non-Gaussian shape of the distribution the distribution-free percentile method (Herrera, 1958), extended by Bezemer (1981) was used to obtain the "estimated inner limit of the upper percentile at P = 0.975 (confidence level γ = 95%)". In a sample of n = 52 observations this percentile value is given by the fourth observation from the largest, which amounted to a trypsin value of 41 U/l. This was rounded to 40 U/l to supply the discrimination limit between negative and positive trypsin values. With this discrimination limit of 40 U/l a positive trypsin test was found in 30 of the 36 animals with oesophagitis and in 4 of the 52 animals without oesophagitis (Fig. 4.11), leading to a sensitivity of the trypsin test of 83% and a specificity of 92%.

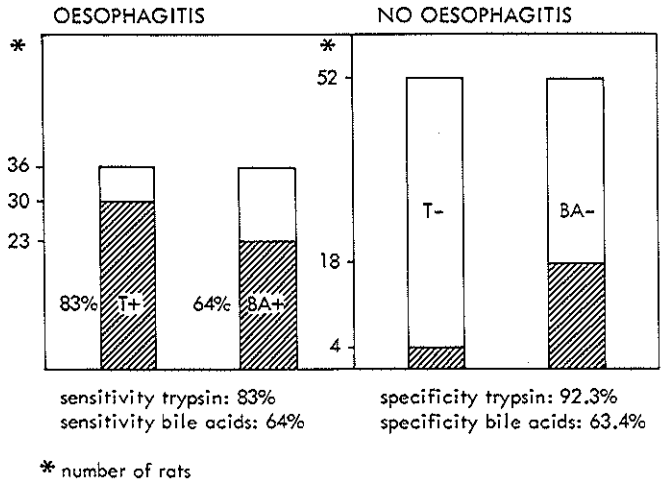


Fig. 4.11. The results of the trypsin and the bile acid test shown as sensitivity and specificity of either test.

4.3.5. Relation of trypsin determination and oesophagitis

The individual post-operative trypsin levels in the oesophagitis group were related to the corresponding macroscopic scores that indicated the severity of the oesophagitis using Spearman's rank correlation test. A correlation between the two variables could not be demonstrated (see Fig. 4.12).

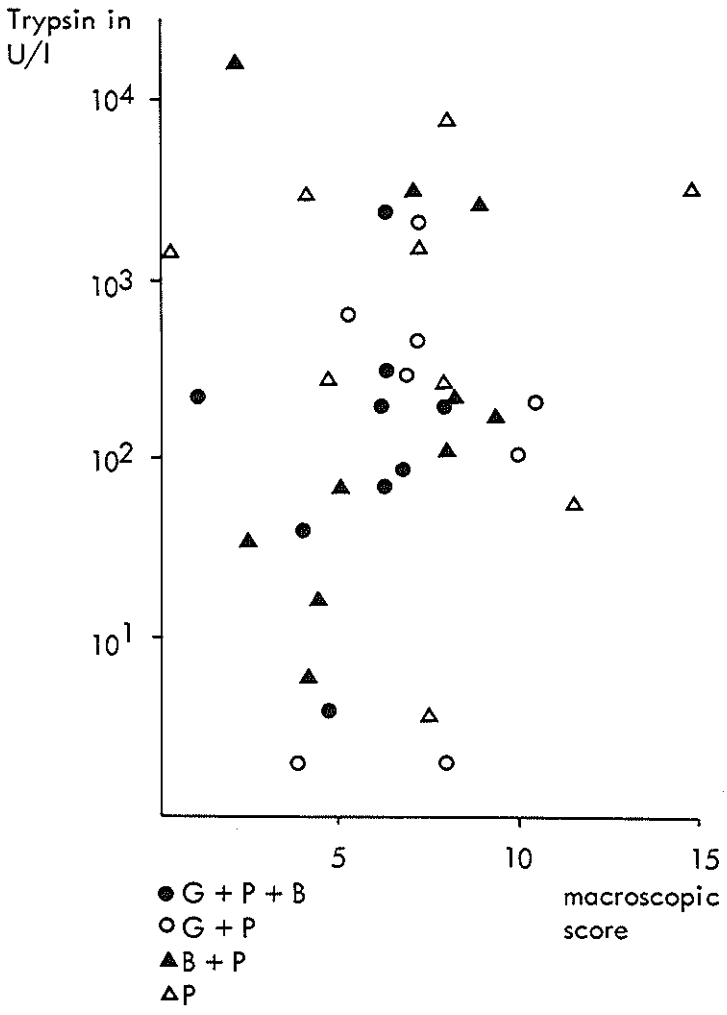


Fig. 4.12. The individual trypsin concentrations in post-operative washout samples per rat with oesophagitis are plotted against the corresponding macroscopic scores.
Spearman's rank correlation test did not show a significant correlation.

4.3.6. Bile acid determinations

The bile acid test used had a lower limit of detection of 10 µmol/l. The result of the bile acid test was called positive if the bile acid concentration was ≥ 10 µmol/l. In all but one pre-operative sample bile acid contents were below this level (Table 4.IV). As to the results of the second samples we found a level of ≥10 µmol/l in 42 rats (range 10-15000 µmol/l) of whom 23 did have and 19 had no oesophagitis leading to a sensitivity of the bile acid test of 64% and a specificity of 63% (Fig. 4.11). The bile acid levels in the second samples of rats with oesophagitis did not differ statistically significant from those without oesophagitis. In the control group the bile acid test in all second samples was negative (Fig. 4.13).

4.3.7. Trypsin, bile acids and oesophagitis

The combined results of the trypsin test and the bile acid test in rats with and without oesophagitis are illustrated in Fig. 4.14. The degree of oesophagitis is expressed by means of the macroscopic score on the vertical axis. Statistical analysis shows no significant difference in the degree of oesophagitis between rats with a positive trypsin test (T+) and a negative bile acid test (BA-) and those with both a positive trypsin and bile acid test. Six rats with oesophagitis had a negative trypsin test. Three of them had a positive bile acid test and belonged to group GBP (1) and group (BP (2). The other remaining three had also a negative bile acid test and belonged to group GP (2) and group P (1).

Table 4.IV. BILE ACID VALUES IN μmol/l

CONTROL GROUPS:

Procedure	n	sample 1 med	sample 1 range	sample 2 med	sample 2 range
Laparotomy	10	<10	<10-14	<10	all <10
Transection	7	<10	all <10	<10	all <10
Roux-en-Y	9	<10	all <10	<10	<10-12
Total	26	<10	<10-14	<10	<10-12

EXPERIMENTAL GROUPS:

No oesophagitis

Type of reflux	n	sample 1 med	sample 1 range	sample 2 med	sample 2 range
G	8	<10	all <10	<10	<10- 220
B	9	<10	all <10	2600	<10-15000
B + G	9	<10	all <10	140	11- 3000
Total	26	<10	all <10	140	<10-15000

Oesophagitis

Type of reflux	n	sample 1 med	sample 1 range	sample 2 med	sample 2 range
G + B + P	9	<10	all <10	300	62- 730
G + P	8	<10	all <10	<10	<10- 240
B + P	10	<10	all <10	330	19-2600
P	9	<10	all <10	<10	<10- 19
Total	36	<10	all <10	70	<10-2600

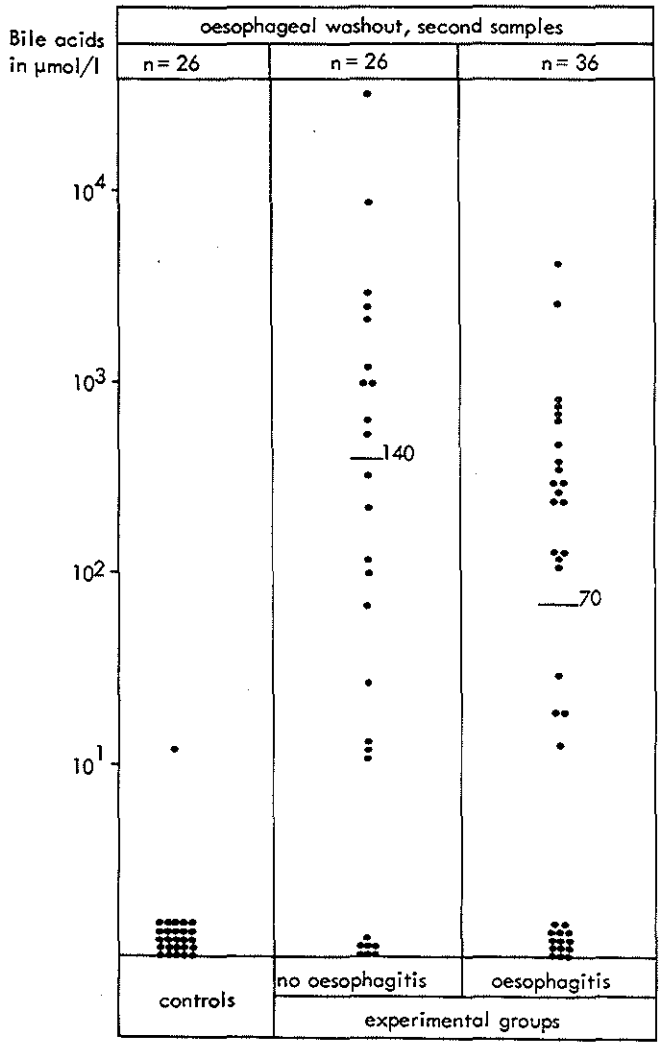


Fig. 4.13. The bile acid concentrations in the post-operative oesophageal washout samples in micromols per liter are shown in a logarithmic scale on the ordinate. Median values are indicated.

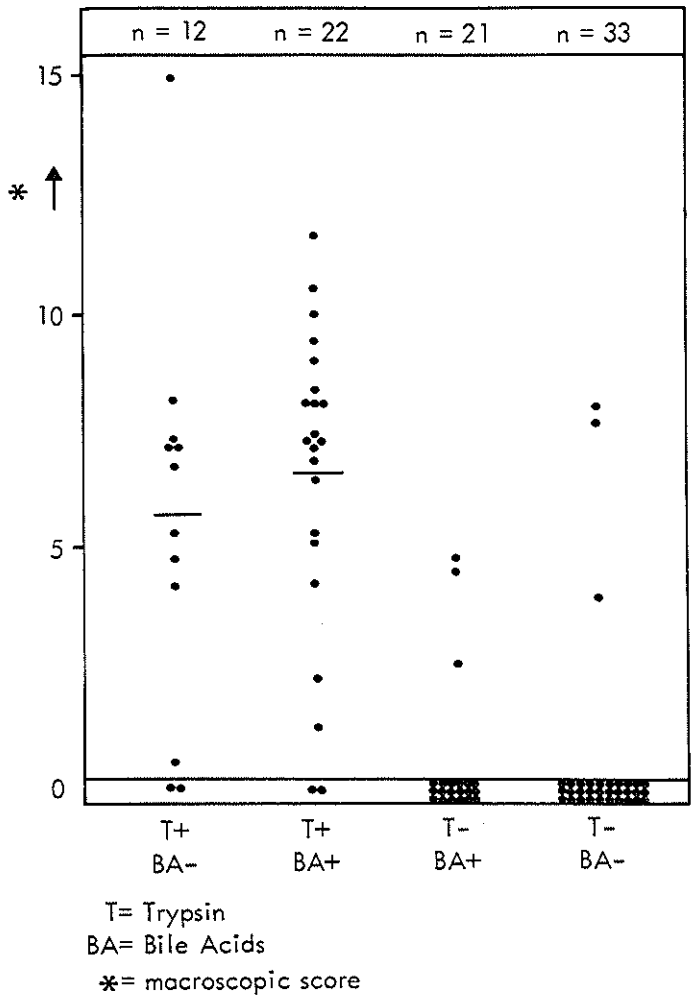


Fig. 4.14. The combined results of the trypsin and the bile acid tests of the post-operative oesophageal washout samples are plotted against the macroscopic scores.
The difference between the first two columns is not statistically significant.
T+ : positive trypsin test
T- : negative trypsin test
BA+: positive bile acid test
BA-: negative bile acid test

4.4. DISCUSSION

In man, reflux of duodenal contents into the oesophagus has been associated with the development of oesophagitis in patients with an intact, functioning stomach (Rovati, 1975; Pellegrini et al., 1978), in patients with an intact, non-secreting stomach (achlorhydria, Palmer, 1960), after a partial gastrectomy (Cox, 1961; Windsor, 1964) and after a total gastrectomy (Helsingen, 1961).

The nature of the offending agent in duodenal juice is subject to controversy. It might originate from bile (bile acids), from pancreatic juice (proteolytic enzymes) or from the combination of bile and pancreatic juice (lysolecithin).

Studies in human volunteers (Safaie-Shirazi et al., 1975) and in rabbits (Chung et al., 1974; Kivilaakso et al., 1980; Harmon et al., 1981) have demonstrated that bile salts in acid solution increase the permeability of oesophageal mucosa to H^+-ions. Kivilaakso et al. (1980) demonstrated in an in vitro study with isolated rabbit oesophageal mucosa that conjugated bile salts (taurocholate) increase the oesophageal mucosal permeability to H^+-ions in acid surroundings and that deconjugated bile salts do so at a neutral pH. Harmon et al. (1981) confirmed these findings in an in vitro rabbit model. The increased permeability to H^+-ions could play a significant role in the pathogenesis of reflux oesophagitis (Kivilaakso, 1980) as it seems to play a significant role in the pathogenesis of gastritis and gastric ulcer (Ritchie, 1977). Bateson et al. (1981) bathed human oesophageal mucosal biopsies, obtained at endoscopy, in a bile acid solution, which resulted in disruption of desmosomes and microvesiculation of cell membranes. These changes increased in number and size with time of exposure and concentration of bile salts and corresponded to those seen in oesophagitis (Hopwood et al., 1979). In perfusion experiments in cats (Cross and Wangensteen, 1951) and dogs (Henderson et al., 1972) bile was shown to cause oesophagitis, while it did not in another perfusion experiment in dogs (Redo, 1959). Surgically induced reflux of bile has been shown to cause oesophagitis in dogs (Cross and Wangensteen, 1951) and in monkeys (Gillison, 1972). However, in none of these experiments the refluent juice has been analysed for its composition, so that the conclusions that have been drawn from these experiments are at least speculative.

Studies in rats by Levrat (1962) implicated bile as the harmful component in surgically induced oesophagitis. Lambert (1962) was able to partially prevent the effects of reflux of duodenal juice into the rat oesophagus by

daily intra-peritoneal administration of a trypsin inhibitor. His conclusion has been that pancreatic juice is the main corrosive agent and that bile can only have a potentiating effect. Kranendonk (1980) has performed rat experiments similar to those of Levrat and Lambert. He found that reflux of pancreatic juice caused oesophagitis and that bile admixture possibly diminished the severity of that oesophagitis.

In the present study the presence of bile acids in the oesophagus was not related to either the occurrence or the degree of oesophagitis. The assay that was used measures total bile acids (Mashige, 1976). Analysis of individual bile acids was not performed. However, it is described that taurocholic acid is the main bile acid in rats (Danielsson et al., 1967), and this has been demonstrated in vitro to be a potentially harmful agent to oesophageal mucosa (Kivilaakso, 1980) as long as free H^+-ions are present. In this experiment data regarding the pH of oesophageal contents are not available, due to the washout technique used, which diluted the oesophageal contents. However, the suggested corrosive action of taurocholate on oesophageal mucosa is not supported by the results of these in vivo experiments.

Cross and Wangensteen (1951) established the potency of pancreatic juice to cause oesophageal lesions in dogs. In vitro experiments with rabbit oesophageal mucosa exposed to trypsin and phospholipase A have demonstrated a damaging effect of trypsin, but not of phospholipase A (Kivilaakso, 1980). Bateson et al. (1981) took oesophageal mucosal biopsies from non-oesophagitis patients and incubated those biopsies in several enzyme solutions. Trypsin in a solution with a pH of 7.2 produced leaking surface cells within 5 minutes; further destruction and cellular debris were noted after longer incubation. The changes produced by trypsin resembled those following incubation of oesophageal mucosa with duodenal juice. Incubation with a lipase solution of neutral pH produced changes comparable to those caused by trypsin.

In rats, the experiments by Lambert (1962) and Kranendonk (1980) have led to the conclusion that reflux of pancreatic juice induces oesophagitis. Furthermore, the finding of Lambert (1962) that oesophageal lesions could be prevented at least partially by daily intra-peritoneal administration of a trypsin inhibitor is an important indication that active trypsin, alone or in combination with other proteolytic enzymes, is an important corrosive factor. The findings in this study establish beyond doubt the relation between the presence of active trypsin in the oesophagus and the occurrence of oesophagitis in rats. However, the results fail to demonstrate a correlation between the concentration of active trypsin in the oesophageal washout samples and the degree of oesophagitis. One or more of the following factors might be responsible for this discrepancy:

1. the presence of reflux at the time of sampling. The samples were taken at various times on the 7th post-operative day; the pattern of reflux was most probably not continuous, and sampling at a time when there was little or no reflux could have resulted in a "false" negative test;
2. the active trypsin concentration in the refluent juice. This concentration is determined by:
   a. the actual exocrine pancreas excretion during the sampling period, which is influenced by many factors, such as the presence of protein (stimulation; Green, 1973), bile (inhibition; Staub and Sarles, 1979) or proteolytic enzymes (inhibition; Green, 1972) in the intestines, and feeding (stimulation; Alphin and Lin, 1959);
   b. the concomitant secretion of gastric juice, bile or enteric juice which introduces an unknown dilution factor. To illustrate this point: in a number of normal non-operated rats duodenal contents have been analysed for active trypsin concentrations which were found in a range from 3800-16260 U/liter;
   c. the intestinal bacterial flora which normally regulates the degradation of proteolytic activity resulting in decreasing activities in an aboral way. Changes might have occurred in the bacterial growth in the different experimental groups with an unknown effect on intestinal trypsin activities;
3. the technical performance of the washout procedure itself. Washout fluid could be lost past the proximal or distal clamp. The presence of food in the oesophagus could further interfere with the adequate mixing of normal saline and oesophageal contents;
4. errors made during storage and subsequent handling of the samples.

The discrimination limit for a positive trypsin test resulted in four "false" positive trypsin tests (2 in control groups and 2 in the bile reflux group), for which there is no simple pathophysiological explanation. Exogenous intake of trypsin with the rat chow is a theoretical possiblity, though several assays of the supernatant of rat chow homogenates never showed any active trypsin. Trypsin-like enzymes are known to be produced by rat salivary glands and could have contaminated oesophageal samples (Rutter et al., 1967), and could also have accounted for the trypsin activity found in all pre-operative samples.

The presence of active trypsin in the oesophageal washout samples is proof of the existence of reflux of pancreatic juice into the oesophagus in the respective experimental models used. Furthermore it is an indication that

a factor in pancreatic juice should be considered as a possible aetiological component in the development of reflux oesophagitis. It could well be that trypsin is that factor, since the pH optimum for trypsin activity, although dependent on the substrate, is between 5 and 8, and the pH in the oesophagus is within this range for the greater part of the day.

The phospholipid lecithin is excreted in bile. Under the influence of the pancreatic enzyme phospholipase A it is converted into lysolecithin, in vitro a mild detergent to oesophageal mucosa (Kivilaakso, 1980). In this study reflux of bile and pancreatic juice together did not cause a more serious oesophagitis than reflux of pancreatic juice alone, so a detergent action of lysolecithin in this experiment seems not very likely.

4.5. SUMMARY AND CONCLUSIONS

In rats, surgically induced reflux of duodenal secretions into the oesophagus may lead to the development of oesophagitis. Different authors have reached divergent conclusions as to the factor or factors in the refluent juice that cause(s) oesophagitis.

Therefore similar rat experiments were performed to investigate whether active trypsin and bile acids are present in the oesophagus of rats before and after a reflux-inducing operation, and whether their presence correlates with the occurrence and degree of oesophagitis.

The results of this study show a statistically significant relation between the presence of active trypsin in the oesophagus and the development of oesophagitis (Fig. 4.10). We found no correlation between the presence of bile acids in the oesophagus and the development of oesophagitis.

There is no evidence of either a potentiating or inhibiting effect of bile on the pancreatic juice related injuries to the oesophageal mucosa.

CHAPTER 5: THE DEVELOPMENT OF MURAL FIBROSIS IN OESOPHAGITIS AND THE EFFECT OF HEALING

5.1. INTRODUCTION

Reflux oesophagitis in man can be a serious and difficult to manage disorder, which may lead to a debilitating oesophageal stricture. Whether or not a given patient will develop a stricture is unpredictable. Histopathologic studies by Sandry (1962, 1972) have demonstrated that a fibrous stricture usually is associated with a severe panmural oesophagitis. This type of oesophagitis is characterized by superficial ulcerative lesions accompanied by severe inflammatory changes of the whole oesophageal wall and sometimes a stenosis. There is always an extensive fibrosis in the submucosa and often a partial destruction of the muscularis mucosae. It is thought that a fibrous stricture is the end-result of the chronic inflammatory process described above (Belsey and Skinner, 1972). Clinically, fibrous strictures can develop in a matter of weeks (Skinner, 1977), but they are usually associated with long-standing symptomatic reflux disease (Ahtaridis, 1979). Also the development of strictures may become apparent when the oesophagitis as such has healed (Westbroek, personal communication). A fibrous stricture is more likely to develop in the presence of mural fibrosis caused by oesophagitis. However, it is not known when this fibrosis starts to develop, and whether or not its development is related to the time during which the reflux oesophagitis has existed. Strictures can be a sequel of "acid" reflux by which is meant reflux in the presence of a normal functioning stomach as well as of "alkaline" reflux, which occurs from a non-secreting stomach in achlorhydria (Palmer, 1960) or after partial gastrectomy (Cox, 1961) or total gastrectomy (Helsingen, 1961).

In rats, surgically induced reflux oesophagitis is a panmural disease with severe inflammatory changes throughout the wall and superficial ulcerative lesions of the epithelium (Levrat et al., 1961; Kranendonk, 1980). Therefore, an investigation was started using surgically induced reflux oesophagitis in rats with the objectives to study the relation between the duration of reflux and the development of mural fibrosis, and furthermore to study the consequences of healing after a reflux abolishing operation.

5.2. MATERIALS AND METHODS

5.2.1. Animals and experimental groups

Male adult Wistar rats were chosen in random order for either a gastric juice, bile and pancreatic juice reflux-inducing operation, or a bile and pancreatic juice reflux-inducing operation. Thus two parallel randomized experimental groups were formed. The rats were exposed to a pre-determined period of reflux and thereafter subjected to a reflux-abolishing operation, or sacrificed to serve as control animals. The observation period after the reflux abolishing operation was again pre-determined. The oesophagus of each rat was washed out at the beginning of the experiment, before the reflux-abolishing operation and before sacrifice following the method described in section 2.6. The pre- and post-operative nursing care of the rats is described in Chapter 2.

5.2.2. Reflux inducing operations

After an oesophageal washout procedure the rats were subjected to an operation to induce reflux of gastric juice (G), bile (B) and pancreatic juice (P) by transection of the intra-abdominal oesophagus and end-to-side oesophagojejunostomy (see Fig. 4.6.) (n = 81), or to induce reflux of bile (B) and pancreatic juice (P) by transection of the intra-abdominal oesophagus followed by total gastrectomy and end-to-side oesophagojejunostomy to the first jejunal loop (see Fig. 4.8.) (n = 75).

5.2.3. Duration of reflux

In both experimental groups the sequential effects of reflux were studied seven, fourteen, twenty-eight or forty-two days after the reflux-inducing operation. After the predetermined period of reflux another oesophageal washout was done and the second sample of the oesophageal contents obtained. Each group of rats that had been subjected to a certain period of reflux was subdivided into four smaller groups. One of these groups served as a control group and the rats belonging to it were exsanguinated after the oesophageal washout. The autopsy procedure will be dealt with in section 5.2.6. The three remaining groups were subjected to a reflux abolishing operation and studied sequentially thereafter (see Fig. 5.1 on unfolding leaf of the cover).

5.2.4. Reflux abolishing operation

The intention of this operation was to divert the contents of the afferent jejunal limb away from the oesophagojejunostomy. For this purpose the afferent limb was ligated close to the oesophagojejunostomy and transected on the duodenal side of the ligature. The afferent limb was then anastomosed to the efferent jejunal limb creating an end-to-side jejunojejunostomy with a Roux loop of 25 cm (see Figs. 5.2 and 5.3). Directly after this Roux-en-Y reconstruction of the oesophagojejunostomy all rats were administered a single dose of 12-15 cc's Ringer's lactate solution subcutaneously between the shoulder blades since a pilot study has shown that in the immediate post-operative period after this second operation most of the rats failed to drink sufficiently, which often resulted in dehydration and death.

5.2.5. Observation of healing

All rats were clinically examined daily and weighed twice a week. They were observed after the reflux abolishing operation for two, four or six weeks respectively. After the pre-determined period of observation following the Roux-en-Y reconstruction the oesophageal washout procedure was repeated and the third sample obtained (see Fig. 5.1). Next a blood sample was taken for haematocrit determination after which the rats were sacrified.

5.2.6. Autopsy

The oesophagus and anastomoses were removed after the length of the Roux loop had been measured. The length of the oesophagus and the width of the anastomosis were determined. The severity of the visible mucosal lesions was registrated with the macroscopic scoring system described in section 2.10. For determination of its collagen content a sample of the oesophageal wall was obtained as described in section 2.11. For histological studies longitudinal sections of each oesophagus were obtained and routinely stained with HAS. Some specimens were stained with AZAN (see section 2.12.1.). Criteria for abnormal findings are described in sections 2.12.2. and 2.12.3.

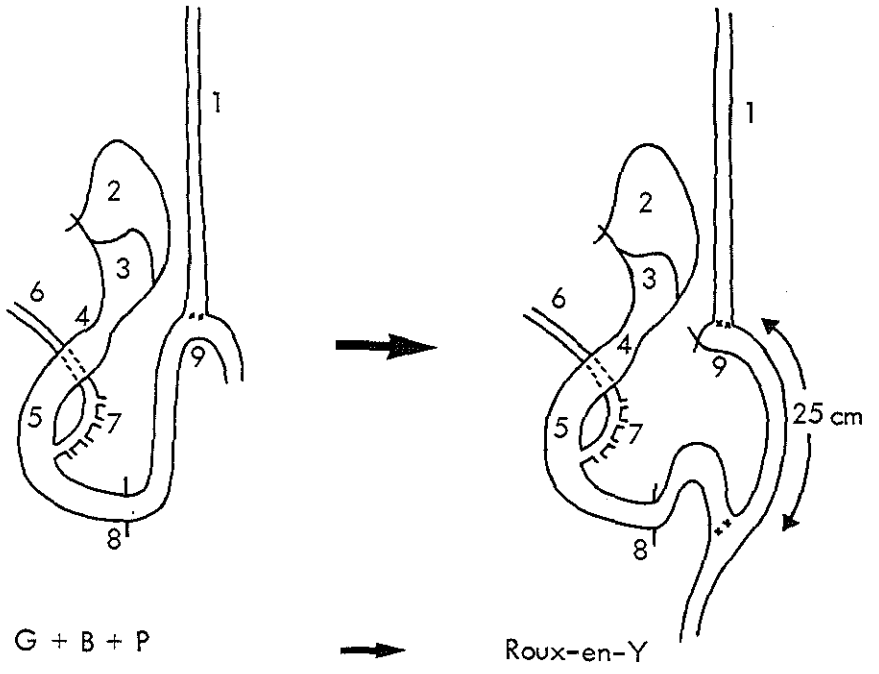


Fig. 5.2. Roux-en-Y diversion of gastric juice (G), bile (B), and pancreatic juice (P).

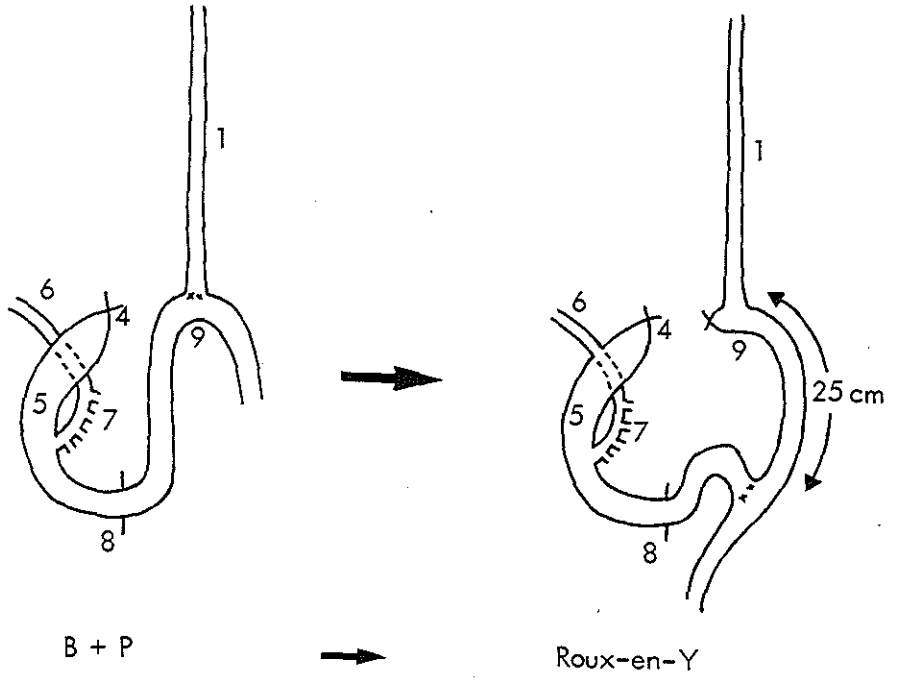


Fig. 5.3. Roux-en-Y diversion of bile (B) and pancreatic juice (P).

5.2.7. Biochemical analysis

5.2.7.1. Oesophageal washout sample

To monitor the induction and later abolition of reflux of active pancreatic juice, the activity of trypsin was determined in all oesophageal wash-out samples with a kinetic method, using S-2160 (Kabi-Vitrum B.V., Amsterdam) as a substrate (Bergström, 1977).

5.2.7.2. Blood sample

Anaemia is a known sequel of gastrectomy in rats. Helsingen and Oystese (1961-III) when studying this subject were unable to prove a relation with the development of oesophagitis. If so, healing of the oesophagitis should result in stabilisation or improvement of the haematocrit level. Therefore the haematocrit was determined in each rat at the end of the experiment. The blood sample, after collection in heparinized glass capillaries, was centrifuged for 10 minutes at 5000 rpm, and the haematocrit determined with a micro-haematocrit reader (Hawksley, England).

5.2.7.3. Oesophageal wall sample

The oesophageal wall sample was analyzed for its collagen content by means of hydroxyproline determinations. Collagen fibres consist of basic units of tropocollagen that are formed by the triple helix of three alpha chains. Each alpha chain is made up of about a thousand amino-acids, to which the following formula can be applied: $(X, Y, Gly)_{333}$. One hundred of the amino-acids on the X-point are proline, one hundred of the amino-acids on the Y-point are hydroxyproline (Gly = glycine). Hydroxyproline is an amino-acid almost unique to collagen. Minute amounts can be detected in elastin, the C 19 sub-component of the complement system, and in the tail of the acetylcholinesterase molecule, but these amounts do no contribute significantly to the hydroxyproline content of a tissue sample (Prockop et al., 1980). Thus, the hydroxyproline content provides an adequate measure for the collagen content of a tissue sample.

Hydroxyproline was determined using a modified version of the Stegemann assay as described by Grant (1964). Collagen content was expressed as a percentage of dry fat free tissue weight using the formula introduced by Neuman and Logan (1950):

$$\frac{\text{hydroxyproline (g)}}{\text{dry fat free tissue weight (g)}} \times 100 \times 7.46$$

5.3. RESULTS

5.3.1. General

A total number of 306 rats was operated upon, of which 156 could be evaluated at the end of the experiment (mortality rate: 49%, see Table 5.I). The reflux-inducing operations carried a mortality rate of 19% due to either technical failures or pneumonia. Four percent of the animals died after they had been exposed to reflux for a few weeks (late deaths). Furthermore, the reflux-abolishing operation was accompanied by a 34% mortality rate. Death was usually due to a combination of cachexia and anaemia. Technical failures were very rare with this operation.

Table 5.I. MORTALITY

Group	Total operated	Early death (0-7 days)		Late death (>7 days)		Death after Roux-en-Y		Total death	
GBP.7	49	12	(24%)	0		17	(35%)	29	(59%)
GBP.14	29	2	(7%)	0		8	(27%)	10	(34%)
GBP.28	42	5	(12%)	3	(7%)	11	(26%)	19	(45%)
GBP.42	35	7	(20%)	4	(11%)	5	(14%)	16	(45%)
Total	155	26	(17%)	7	(5%)	41	(26%)	74	(48%)
BP.7	44	10	(23%)	0		15	(34%)	25	(57%)
BP.14	39	9	(23%)	0		10	(26%)	19	(49%)
BP.28	35	7	(20%)	2	(6%)	8	(23%)	17	(49%)
BP.42	33	7	(21%)	3	(9%)	5	(15%)	15	(45%)
Total	151	33	(22%)	5	(3%)	28	(25%)	76	(50%)
Grand total	306	59	(19%)	12	(4%)	79	(26%)	150	(49%)

All percentages in the table are related to the total number of rats operated upon in that group.

5.3.2. Weight

All animals lost weight after the reflux inducing operation, some up to over 30% of their pre-operative weight. After the reflux-abolishing operation a slow but steady weight gain was observed. These findings are illustrated in Figs. 5.4 and 5.5.

5.3.3. Length of the Roux-loop

The Roux-loop tended to be a bit longer than planned. The mean length was 33 cm, range 21-50 cm.

5.3.4. Length of the oesophagus

In rats that were studied as control groups following a period of reflux we found a shorter oesophagus after a longer period of reflux. In rats that were studied after a Roux-en-Y reflux-abolishing operation the oesophageal length was usually larger than that of their control group (Table 5.II).

Table 5.II. MEAN LENGTH OF THE OESOPHAGUS IN cm ± SEM

Group	Weeks after Roux-en-Y			
	C	2	4	6
GBP. 7	7.0 ± 0.1	7.5 ± 0.1	7.6 ± 1.3	7.2 ± 0.2
GBP.14	7.1 ± 0.2	7.4 ± 0.2	6.8 ± 0.5	7.5 ± 0.1
GBP.28	6.2 ± 0.2	6.8 ± 0.2	7.1 ± 0.1	7.1 ± 0.1
GBP.42	5.6 ± 0.2	6.9 ± 0.3	6.6 ± 0.4	6.8 ± 0.3
BP. 7	7.0 ± 0.4	7.5 ± 0.3	7.5 ± 0.3	7.7 ± 0.2
BP. 14	6.9 ± 0.3	7.1 ± 0.3	7.1 ± 0.4	6.7 ± 0.3
BP. 28	5.8 ± 0.4	6.8 ± 0.5	6.7 ± 0.3	6.6 ± 0.4
BP. 42	5.8 ± 0.2	6.0 ± 0.3	6.6 ± 0.4	7.2 ± 0.2

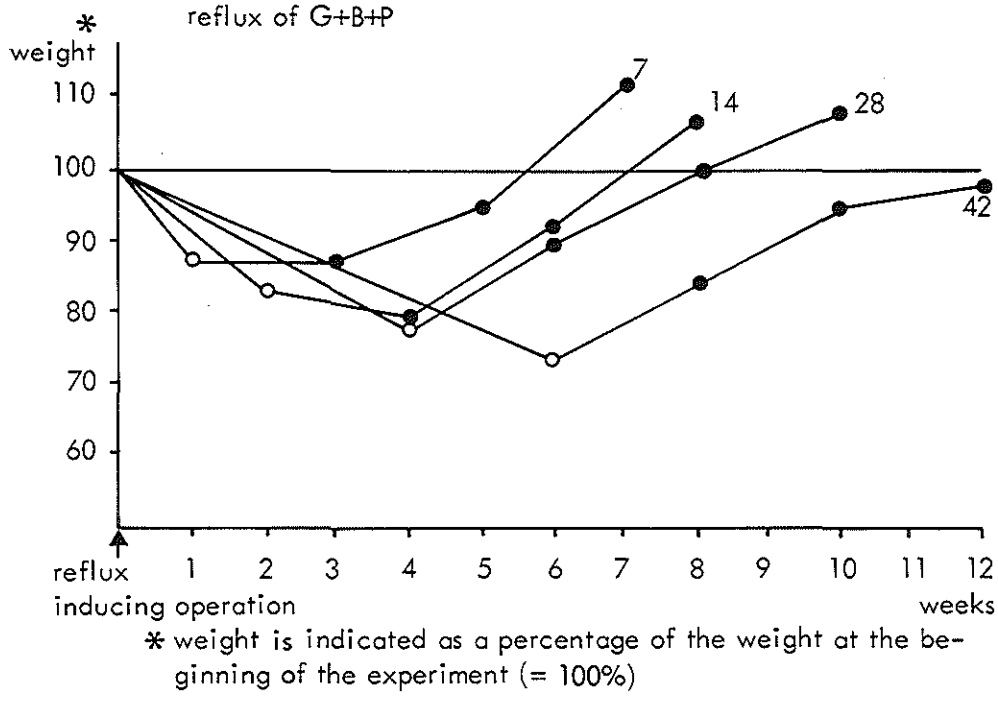


Fig. 5.4. Weight curve of group GBP. The dots indicate the mean weight of rats studied at that moment in the experiment. Open dots indicate the reflux-abolishing operation.

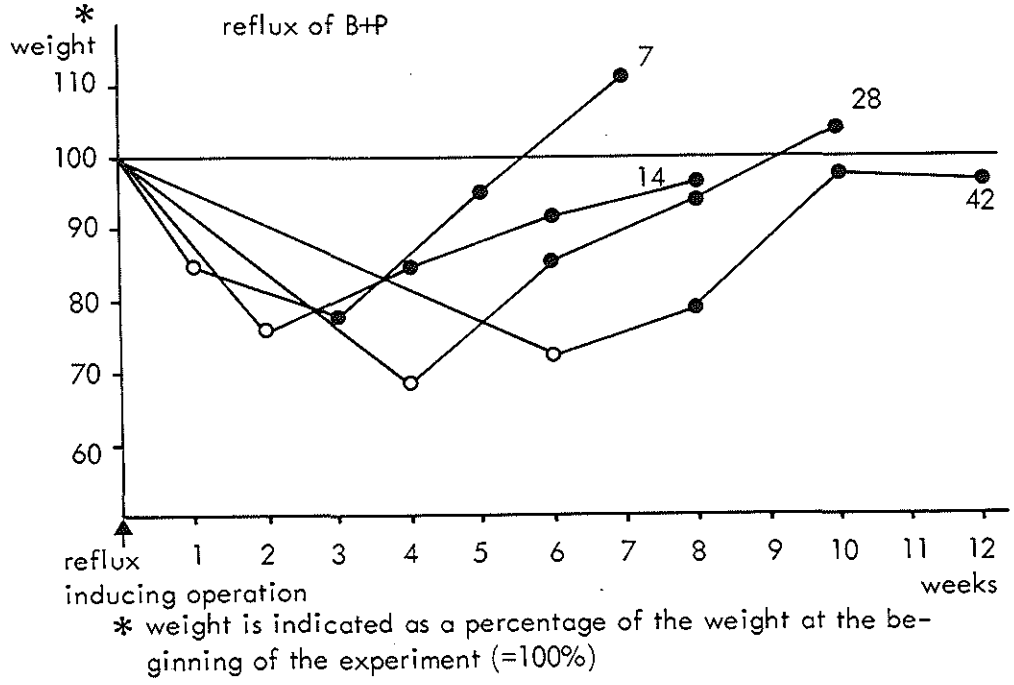


Fig. 5.5. Weight curves of groups BP. The dots indicate the mean weight of rats studied at that moment in the experiment. Open dots indicate the reflux-abolishing operation.

5.3.5. Macroscopic examination

All rats that were killed after the reflux-inducing operation had macroscopically distinct oesophagitis. More extensive lesions were seen when the rats had been subjected to a longer period of reflux. In none of the rats oesophageal strictures were encountered. After the reflux-abolishing operation the lesions gradually disappeared in time (see Table 5.III).

Table 5.III. MEAN MACROSCOPIC SCORE OF OESOPHAGEAL LESIONS BEFORE AND AFTER REFLUX-ABOLISHING OPERATION

Group GBP	n_{oes}/n_{ex}	$\bar{x}$	Group BP	$n_{oes}n/_{ex}$	$\bar{x}$	Total
7.C	5/ 5	6.5	7.C	5/ 5	4.9	
14.C	4/ 4	8.6	14.C	5/ 5	7.5	
28.C	8/ 8	9.9	28.C	5/ 5	11.9	
42.C	5/ 5	9.9	42.C	4/ 4	12	
Total	22/22			19/19		41/41
7.2	1/ 4	1	7.2	1/ 4	0.1	
14.2	5/ 6	4.7	14.2	1/ 4	1.7	
28.2	5/ 5	7.5	28.2	3/ 4	3.4	
42.2	3/ 4	5.4	42.2	3/ 4	11.5	
Total	14/19			8/16		22/35
7.4	0/ 5	-	7.4	0/ 5	-	
14.4	2/ 5	1.6	14.4	3/ 6	0.7	
28.4	0/ 5	-	28.4	2/ 4	1.6	
42.4	0/ 5	-	42.4	0/ 5	-	
Total	2/20			5/20		7/40
7.6	0/ 6	-	7.6	0/ 5	-	
14.6	0/ 4	-	14.6	1/ 5	1.2	
28.6	0/ 5	-	28.6	0/ 5	-	
42.6	0/ 5	-	42.6	0/ 5	-	
Total	0/20			1/20		1/40

n_{oes} = number of rats with oesophagitis
n_{ex} = number of rats in the experimental group
$\bar{x}$ = mean macroscopic score

Fourteen days after Roux-en-Y reconstruction abnormalities were seen in 22 out of 35 rats studied. Four weeks after Roux-en-Y reconstruction abnormalities were seen in 7 out of 40 rats studied. After six weeks the oesophagus was normal in all 40 rats but one. In this single rat, the mucosa was thought to be erosive on macroscopic examination and allotted a score of 6.1. However, the microscopic examination of the oesophagus revealed a normal mucosa.

5.3.6. Microscopic study

In Figure 5.6 two sections of the oesophagus of non-operated rats are presented to demonstrate the normal histological picture.

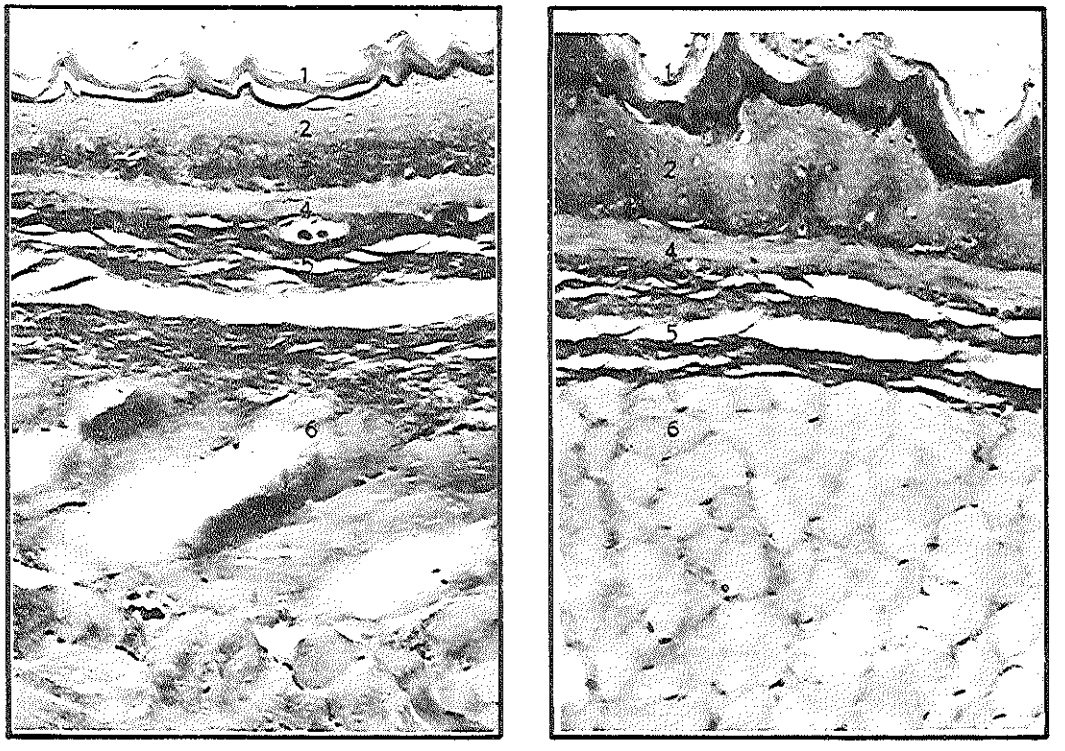


Fig. 5.6. Two examples of a normal oesophageal wall.
1. keratin layer; 2. squamous epithelium; 3. tunica propria;
4. musc. mucosae; 5. submucosa; 6. musc. propria.

5.3.6.1. General

All rats that served as controls after the reflux inducing operations had microscopically severe oesophagitis. The epithelial lesions were erosive after one week of reflux and became more and more ulcerative with the increasing duration of reflux. After 14 days also hyperkeratotic lesions appeared. The Roux-en-Y reconstruction resulted in repair of the epithelial layer in almost all rats within 2 weeks. Of course, hyperkeratosis and hyperplasia were still frequently encountered, but erosive or ulcerative lesions had virtually disappeared.

5.3.6.2. Microscopic description per group

The results are presented per experimental group, starting with the histological findings after a certain period of reflux, followed by the sequential findings two, four, and six weeks after the Roux-en-Y operation. Parallel GBP and BP groups are presented together.

GBP.7.C.: mainly erosive (Fig. 5.7) and some ulcerative (Fig. 5.8) lesions are seen in the distal half of the oesophagus. The tunica propria and submucosa are densely infiltrated with polymorphonuclear cells. The infiltrate gradually fades towards the perifery of the oesophagus. In the transition zone between erosive and normal epithelium hyperplasia of basal cells is seen. Hyperkeratosis (Fig. 5.9) is seen only once.

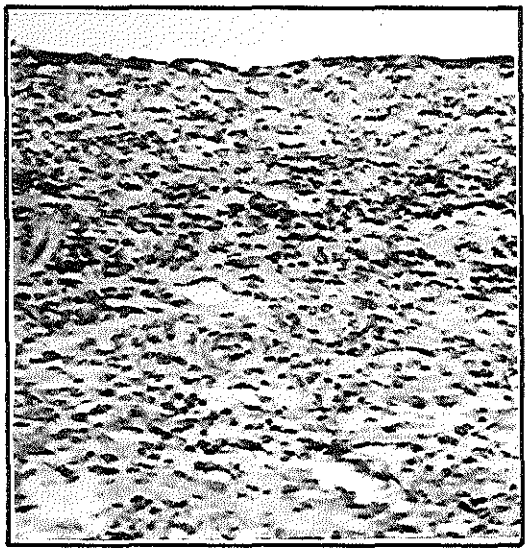


Fig. 5.7. Erosive epithelium (GBP.42.C)

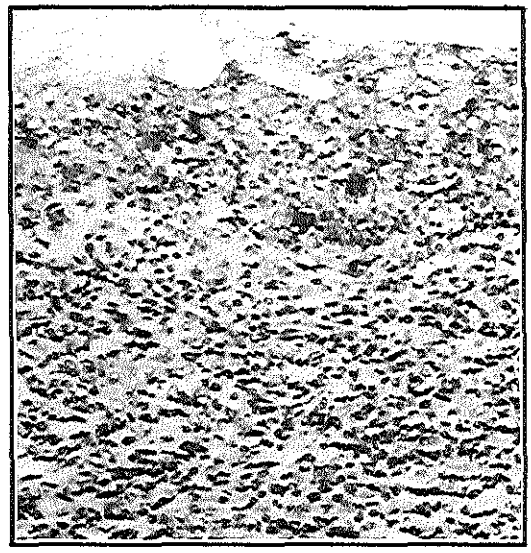


Fig. 5.8. Ulcerative epithelium (GBP.42.C)

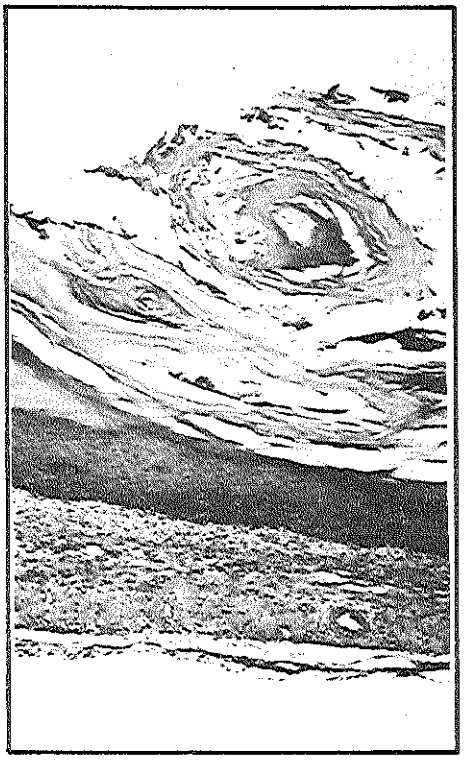


Fig. 5.9. Hyperkeratosis (GBP.14.2).

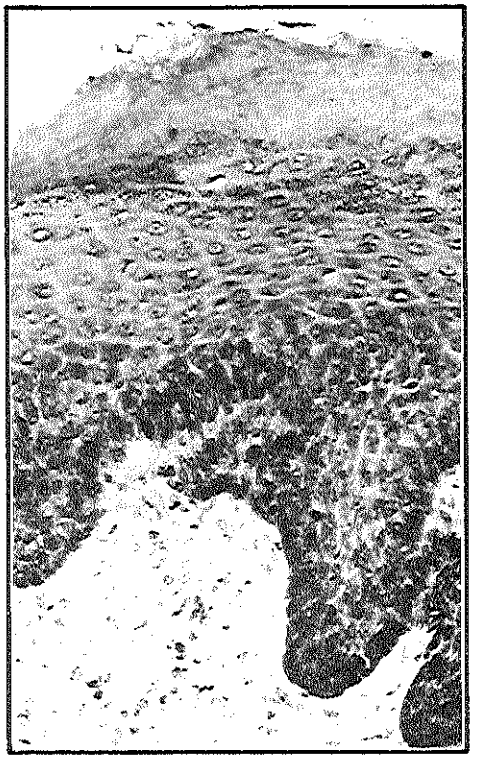


Fig. 5.10. Hyperplasia of the epithelium (GBP.42.4).

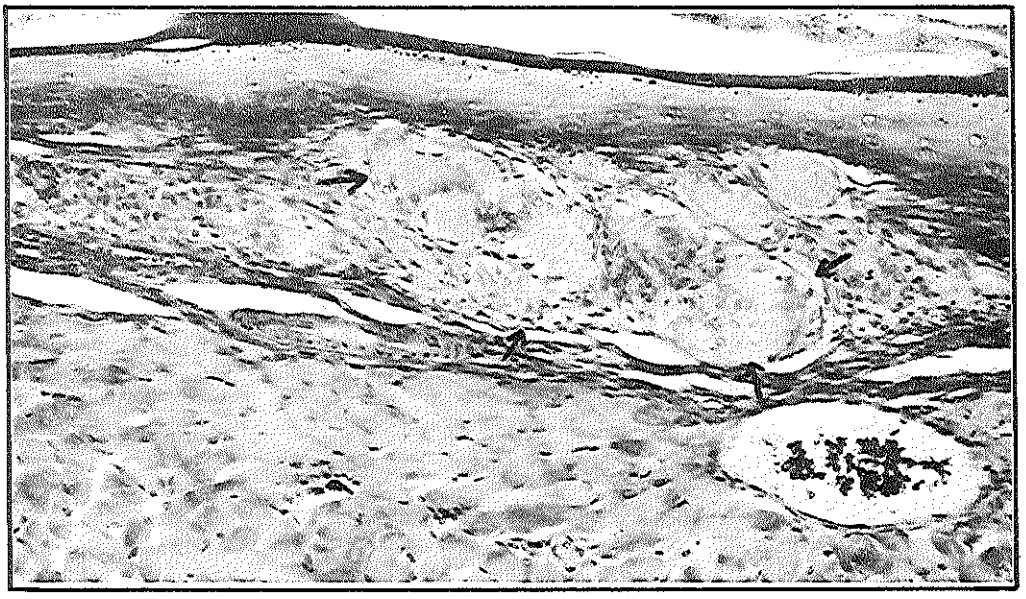


Fig. 5.11. Example of large granuloma's in the submucosa at arrows (GBP.42.4).

BP.7.C.: as in GBP.7.C.

After Roux-en-Y operation:

GBP.7.2: the epithelium is intact throughout the oesophagus and shows moderate hyperplasia in places (Fig. 5.10). There is some basal cell hyperplasia. Accumulations of round cells are seen scattered throughout the tunica propria and submucosa. The muscularis mucosae is often interrupted and sometimes hypertrophic. The muscularis propria and adventitia are normal.

BP. 7.2: as in GBP.7.2. In the submucosa some granuloma's due to food particles or hair are visible (Fig. 5.11).

GBP.7.4: the epithelium is normal. Interruptions of the muscularis mucosae are still present as well as hypertrophic areas. In the submucosa granuloma's are seen as well as a few round cells. The muscularis propria and adventitia are normal.

BP. 7.4: as in GBP.7.4. In one rat the submucosa shows collections of round cells.

GBP.7.6: the oesophagus is normal apart from scattered interruptions and areas of hypertrophy in the submucosa.

BP. 7.6: as in GBP.7.6.

GBP.14.C: the distal half of the oesophagus is ulcerative. In some rats there is hyperplastic epithelium with hyperkeratosis and increased height of the rete pegs. The epithelium at the top of the rete pegs is often atrophic or completely absent. The oesophageal wall is densely infiltrated with polymorphonuclear cells obscuring the normal anatomy of the oesophagus. In the mucosa there is an increase of fibrinoid material. Perioesophageally some inflammatory cells and dilated vessels are seen.

BP. 14.C: as in GBP.14.C.

After Roux-en-Y operation:

GBP.14.2: the epithelium is intact but shows hyperplasia, hyperkeratosis and increased height of the rete pegs. The muscularis mucosae is frequently interrupted and in other places hypertrophic. In the submucosa collections of round cells are present as well as granuloma's. The muscularis propria is normal. Perioesophageally collections of round cells are seen around food particles.

BP. 14.2: as in GBP.14.2. In one rat the oesophagus is erosive in one place.

GBP.14.4: an intact epithelium is seen that shows occasional hyperplasia and hyperkeratosis. The muscularis mucosae shows infrequent patches of hypertrophy and is sometimes absent. In one rat there is still a substantial round cell infiltrate throughout the mucosa, whereas in all other specimens there are granuloma's and circumscript concentrations of round cells. The muscularis propria and adventitia are normal.

BP. 14.4: apart from a slight hyperkeratosis the findings are similar to those in group GBP.14.4.

GBP.14.6: the oesophagus is normal with occasional hypertrophic areas in the muscularis mucosae.

BP. 14.6: as in GBP.14.6, plus a few granuloma's in the submucosa.

GBP.28.C: extensive ulcerations alternate with areas of hyperplasia and hyperkeratosis. There is a dense inflammatory infiltration of the wall consisting of polymorphonuclear and round cells. In the submucosa there is ample fibrinoid material. Only the most proximal part of the oesophagus is normal.

BP. 28.C: as in GBP.28.C.

After Roux-en-Y operation:

GBP.28.2: the epithelium is intact, apart from a small ulcerative lesion in one specimen. It does, however, show hyperplasia and hyperkeratosis in many places, as well as an increased height of rete pegs. In both tunica propria and submucosa patches of round cells are found. The muscularis mucosae is often hypertrophic and sometimes absent. The muscularis propria is thin in places. The adventitia shows some fibrosis.

BP. 28.2: as in GBP.28.2.

GBP.28.4: the epithelium is intact with sometimes a slight hyperkeratosis. There are few inflammatory cells. Some hypertrophic areas are visible in the muscularis mucosae. There is an occasional granuloma in the submucosa. The muscularis propria and adventitia are normal.

BP. 28.4: as in GBP.28.4.

GBP.28.6: the epithelium is normal except for one specimen in which there is occasional hyperkeratosis. The tunica propria is normal. The muscularis mucosae is present over large distances and is rarely hypertrophic. In one specimen there are striated muscle-like structures visible in the submucosa. The muscularis propria and adventitia are normal.

BP. 28.6: as in GBP.28.6.

GBP.42.C: a large part of the laminal surface of the oesophagus is ulcerative with a few hyperkeratotic islands remaining. There is a dense transmural, chiefly polymorphonuclear infiltration throughout the wall, as well as fibrinoid deposits. Perioesophageally there are dilated vessels and collections of round cells.

BP. 42.C: as in GBP.42.C.

After Roux-en-Y operation:

GBP.42.2: an intact though hyperkeratotic epithelium is seen in all specimens. Accumulation of round cells appear scattered throughout the submucosa. The muscularis mucosae is often interrupted or hyperplastic. The muscularis propria is normal.There is some perioesophageal reaction.

BP. 42.2: as in GBP.42.2. In one specimen there is a sealed perforation of the wall as well as a slight overgrowth of the oesophagus by jejunal epithelium.

GBP.42.4: the epithelium is intact with patches of hyperkeratosis. There are but few inflammatory cells in the tunica propria and the submucosa. The muscularis mucosae is normal in places, but sometimes hypertrophied or interrupted. The muscularis propria and adventitia are normal. In the submucosa there are some granuloma's.

BP. 42.4: as in GBP.42.4.

GBP.42.6: the epithelium is normal. The sometimes interrupted (Fig. 5.12) muscularis mucosae is hypertrophic (Fig. 5.13) in places. The amount of fibroid tissue in the tunica propria and submucosa is possibly slightly increased. The muscularis propria and adventitia are normal.

BP. 42.6: as in GBP.42.6.

In summary, the results show an increasingly severe oesophagitis the longer the period of reflux had been. In virtually all rats studied two weeks after the Roux-en-Y reflux-abolishing operation, the epithelium was intact albeit hypertrophic. Thereafter the epithelium as well as the other layers of the oesophageal wall gradually returned to normal.

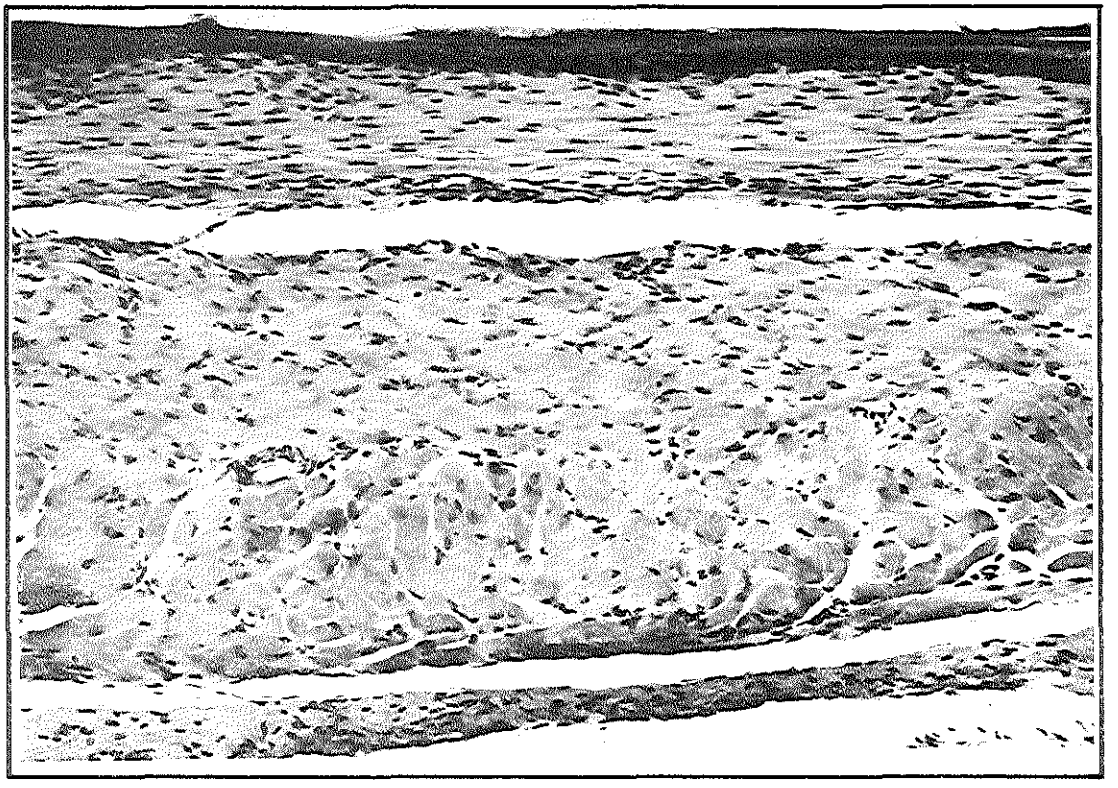


Fig. 5.12. The oesophagus six weeks after the reflux of pancreatic juice was terminated. The muscularis mucosae is absent (GBP.42.6).

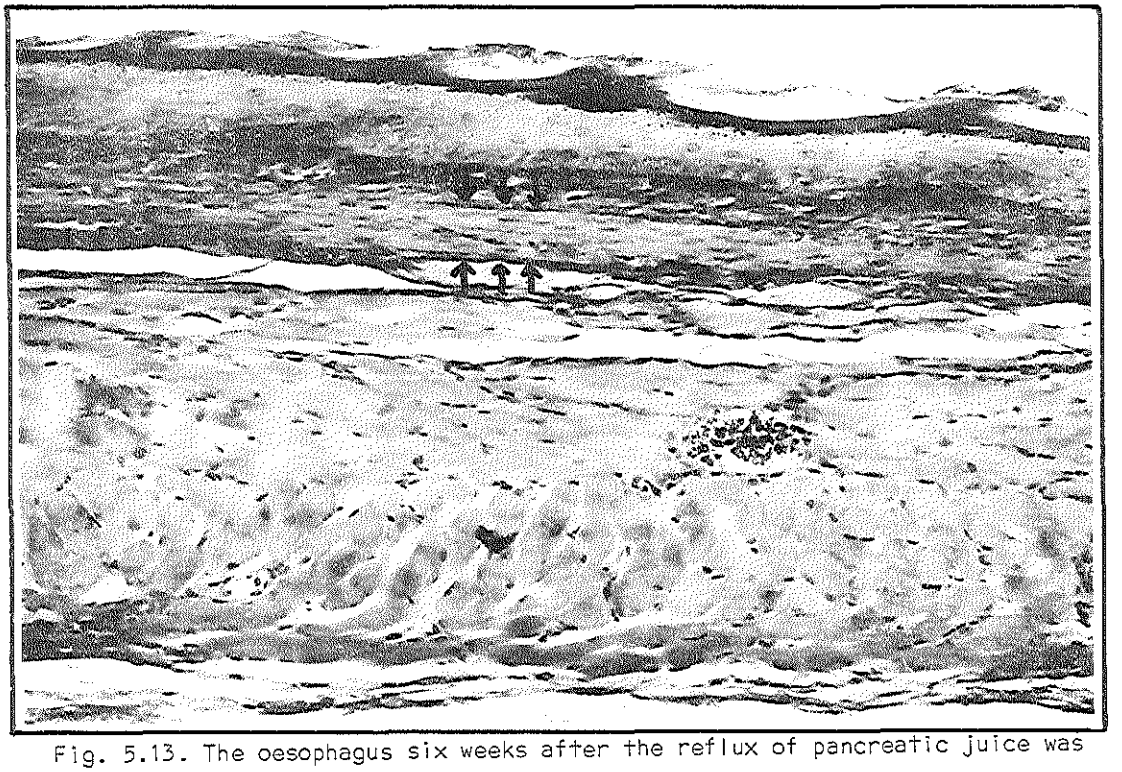


Fig. 5.13. The oesophagus six weeks after the reflux of pancreatic juice was terminated. The muscularis mucosae (arrows) shows marked hyperplasia (GBP.42.6).

5.3.6.3. Microscopic assessment of fibrosis

An attempt was made to quantify fibrosis in the microscopic sections stained with azocarmine and anilin (AZAN). A number of sections was coded and stained, and examined by the author. Thickness of the lamina propria and submucosa was measured in each section, one, two and three cm proximal to the oesophagojejunostomy. After breaking of the code the results were subjected to statistical analysis. Significant differences could not be demonstrated (Table 5.IV). In view of these findings further attempts to microscopic quantification of fibrosis were abandoned.

5.3.7. Trypsin determinations

In group GBP the mean value in the first sample was 1 U/l (range 0-5), in the second sample 881 U/l (range 7-6800) and in the third sample 4 U/l (range 0-30).

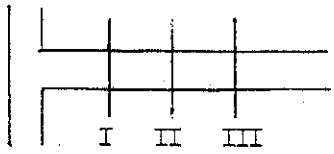
In group BP the mean value in the first sample was 2 U/l (range 0-10), in the second sample 645 U/l (range 0-7250) and in the third sample 19 U/l (range 0-425).

The mean results per sub-group are shown in Figs. 5.14 and 5.15. The results show that a reflux inducing operation was associated with a significant rise in active trypsin levels in the oesophageal wash-out samples. After the reflux-abolishing operation trypsin levels came down to a very low concentration again. In four rats there still was a rather high trypsin level in the oesophagus after the reflux-abolishing operation. In one rat this was associated with persisting severe oesophagitis 6 weeks after Roux-en-Y diversion. This rat was moved from the study since the reflux-abolishing operation had apparently missed its goal.

5.3.8. Haematocrit determinations

The normal value of haematocrit in rats in our laboratory is 52%, range 50-54%. Virtually all rats were clinically anaemic with decreasing haematocrit levels when the duration of reflux had been longer (Table 5.V). The healing of oesophagitis after Roux-en-Y diversion was not associated with improvement of the haematocrit percentages.

Table 5.IV. MICROSCOPIC QUANTIFICATION OF FIBROSIS

jejunum  oesophagus

Group	Place of measurements×:						Wilcoxon's rank sum test
	Tunica propria			Submucosa			
	I	II	III	I	II	III	
Non-operated	4	6	7	10	15	16	
control rats	6	8	8	-	10	14	
	6	5	5	15	13	12	
	3	4	7	9	7	17	
	5	4	6	18	7	13	
GBP. 7.2	3	4	6	10	12	40	
	2	-	-	5	-	-	
GBP. 7.6	8	6	8	25	30	8	
	2	2	6	6	8	20	
	4	5	12	9	10	12	
	4	9	19	10	15	25	
	ns	ns	ns	ns	ns	ns	GBP.7.6 vs control rats
	ns	ns	ns	ns	ns	ns	GBP.7.2+GBP.7.6 vs control rats
GBP.42.2	4	4	10	7	10	30	
	4	7	5	7	10	12	
	5	3	3	8	11	10	
GBP.42.6	5	5	-	11	9	-	
	6	10	-	8	5	-	
	13	5	3	30	14	7	
	2	5	8	10	8	9	
	ns	ns	-	ns	ns	-	GBP.42.6 vs control rats
	ns	ns	ns	ns	ns	ns	GBP.42.2+GBP.42.6 vs control rats

× The measurements are in ocular calibrations.

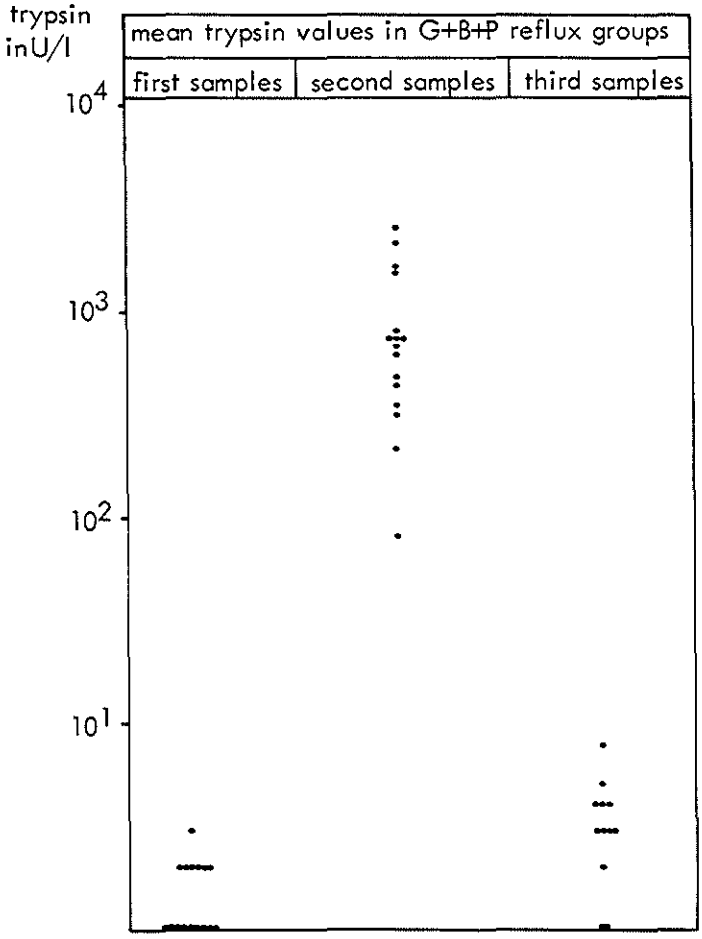


Fig. 5.14. Mean trypsin levels per GBP group in the oesophageal washout samples.

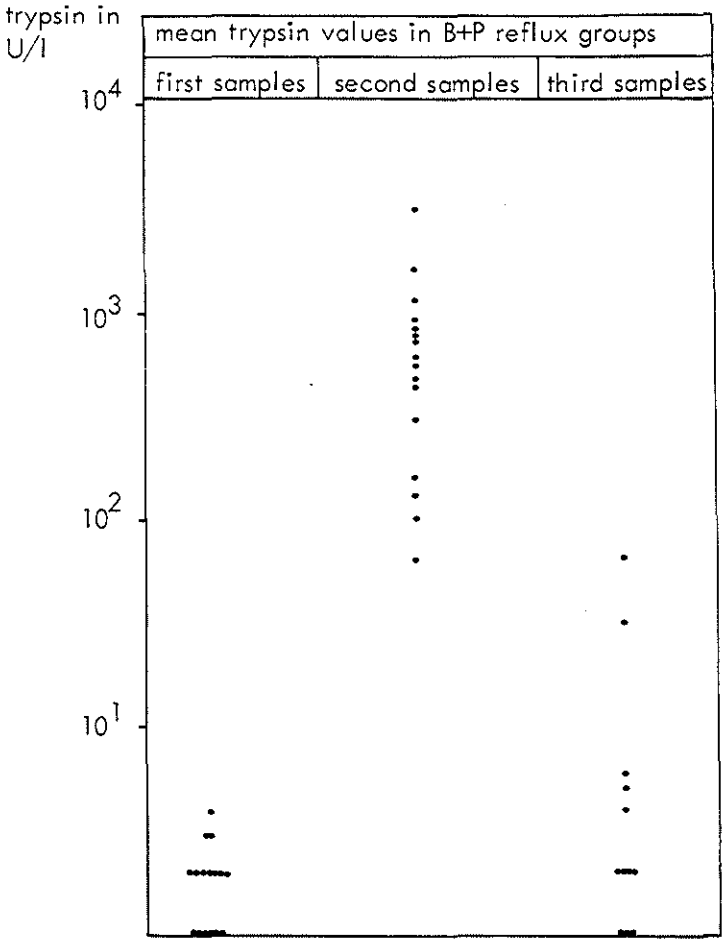


Fig. 5.15. Mean trypsin values per BP group in the oesophageal washout samples.

Table 5.V. HAEMATOCRIT DETERMINATIONS (mean % ± SEM)

Group	Weeks after Roux-en-Y			
	C	2	4	6
GBP. 7	55 ± 2	38 ± 6	38 ± 4	34 ± 3
GBP.14	41 ± 4	34 ± 3	25 ± 3	23 ± 5
GBP.28	31 ± 4	32 ± 2	26 ± 2	28 ± 1
GBP.42	27 ± 7	24 ± 2	24 ± 3	25 ± 1
BP. 7	27 ± 3	48 ± 3	39 ± 4	41 ± 1
BP. 14	37 ± 3	36 ± 6	34 ± 4	35 ± 5
BP. 28	38 ± 8	30 ± 4	36 ± 1	33 ± 3
BP. 42	39 ± 3	28 ± 3	30 ± 1	30 ± 2

5.3.9. Collagen content determinations

5.3.9.1. General results

A total number of 165 samples was collected for collagen content determination; 15 samples were lost in a malfunctioning refrigerator and another 12 during the processing of the samples. Nine samples were obtained from normal non-operated rats to serve as control values.

5.3.9.2. Collagen content and dry fat free tissue weight per experimental group after GBP and BP reflux

The results are presented in Tables 5.VI and 5.VII. Collagen content is expressed as a percentage of dry fat free tissue weight. The tables also present data concerning the dry fat free tissue weight expressed as a percentage of wet tissue weight and further abbreviated as DFFT. The induction of reflux was associated with a decrease of DFFT which became more pronounced when the duration of reflux had been longer. After the Roux-en-Y diversion a gradual return of DFFT to normal values occurred. The changes in DFFT are interpreted as a presentation of the increase and decrease of inflammatory oedema.

5.3.9.3. Combined results of the collagen content determinations (GBP+BP)

The results of the experiments that were described in Chapters 3 and 4 of this thesis endorse the assumption that there is no essential difference in the composition of the refluent juice in groups GBP and BP. It was therefore thought justifiable to combine the results of the collagen content determinations of both reflux groups. The combined results per experimental group were compared with the results of the control group for statistical analysis and are presented in table 5.VIII. A significantly higher collagen content was found in the following groups:

(GBP + BP)	7.6	($p < 0.05$)
(GBP + BP)	28.6	($p < 0.01$)
(GBP + BP)	42.C	($p < 0.05$)
(GBP + BP)	42.4	($p < 0.05$)
(GBP + BP)	42.6	($p < 0.01$)

The statistical analysis of the combined results seems to indicate a relation between the collagen content of the oesophageal wall and on the one hand the duration of reflux, and on the other hand the time elapsed since the Roux-en-Y procedure (Fig. 5.16). Comparison of all control values in the experi-

Table 5.VI. COLLAGEN DETERMINATIONS IN OESOPHAGEAL WALL SAMPLES AFTER G + B + P REFLUX

Group	$\frac{\text{dry fat free tissue (mg)}}{\text{wet tissue (mg)}} \times 100$			% collagen of dry fat free tissue		
	n	$\bar{x}$	Range	n	$\bar{x}$	Range
Control	9	19.9	17.8-22.7	9	14.5	11.3-17.1
GBP.7.C	3	16.9	15.2-18.8	2	14.5	11.2-17.8
GBP.7.2	4	16.7	8.3-20.0	4	12.7	10.8-16.4
GBP.7.4	5	17.5	12.0-20.0	4	14.2	10.2-19.3
GBP.7.6	5	19.7	15.9-22.4	4	19.0	12.9-22.1
GBP.14.C	2	15.4	14.5-16.2	2	15.6	10.9-20.3
GBP.14.2	4	16.0	6.7-20.0	3	13.4	12.0-15.0
GBP.14.4	5	19.6	16.4-23.3	3	14.4	11.7-18.2
GBP.14.6	3	19.8	18.6-20.7	3	18.7	14.8-20.6
GBP.28.C	7	15.7	11.9-20.9	7	17.2	11.0-28.0
GBP.28.2	3	17.1	15.4-18.5	3	16.2	8.5-22.6
GBP.28.4	5	19.3	18.7-20.0	4	17.3	14.9-21.4
GBP.28.6	5	21.1	18.8-23.7	3	23.5	23.0-23.9
GBP.42.C	5	13.1	7.2-15.9	5	17.1	11.8-20.1
GBP.42.2	4	17.3	16.4-18.0	4	14.9	7.3-20.9
GBP.42.4	5	19.3	17.9-20.0	3	18.3	15.8-22.4
GBP.42.6	5	19.9	17.0-21.8	2	21.7	19.6-23.7

mental groups with different periods of reflux showed no significant difference (Kruskal and Wallis). Also no significant differences were demonstrated when comparing the collagen content values after different periods of reflux two, four, and six weeks after Roux-en-Y diversion, respectively. The graphical representation of the results (Fig. 5.17) demonstrated for every period of reflux a biphasic curve of the results of the collagen content determinations illustrating the influence of the abolishment of reflux and subsequent healing. The combined results (GBP + BP) of each reflux group were statistically examined for evidence of significant differences between the various subgroups as determined by the time interval between the termination of reflux and the

Table 5.VII. COLLAGEN DETERMINATIONS IN OESOPHAGEAL WALL SAMPLES AFTER B + P REFLUX

	$\frac{\text{dry fat free tissue (mg)}}{\text{wet tissue (mg)}} \times 100$			% collagen of dry fat free tissue		
Group	n	$\bar{x}$	Range	n	$\bar{x}$	Range
Control	9	19.9	17.8-22.7	9	14.5	11.3-17.1
BP.7.C	5	16.4	14.7-17.2	4	15.9	14.7-18.2
BP.7.2	3	20.8	19.2-22.5	4	14.4	13.2-16.7
BP.7.4	4	19.8	17.2-22.1	4	14.8	7.3-22.2
BP.7.6	5	18.7	17.3-20.0	5	18.4	15.0-24.5
BP.14.C	4	15.3	14.3-16.5	4	13.2	10.9-17.1
BP.14.2	4	18.3	15.0-20.0	3	13.1	8.7-16.1
BP.14.4	6	19.2	17.7-21.1	5	17.3	14.6-20.1
BP.14.6	4	19.8	17.9-22.0	4	18.5	6.1-22.6
BP.28.C	3	15.2	13.3-16.5	3	17.2	14.0-21.1
BP.28.2	4	15.2	9.1-19.4	3	11.8	8.6-13.7
BP.28.4	4	19.3	17.6-20.6	4	18.1	10.3-22.7
BP.28.6	5	18.8	16.2-22.0	1	-	21.5
BP.42.C	4	15.7	14.8-16.7	4	18.5	13.8-22.8
BP.42.2	4	20.0	18.9-22.1	4	15.3	11.1-20.0
BP.42.4	5	18.1	15.9-20.6	4	18.1	11.7-22.9
BP.42.6	5	19.0	18.3-19.4	3	23.7	20.7-28.2

acquisition of the oesophageal wall sample. Comparison of the results obtained directly after seven days of reflux and two, four and six weeks after Roux-en-Y diversion (Fig. 5.18) showed a signifcant difference between samples collected two and six weeks after Roux-en-Y diversion ($p < 0.02$, Wilcoxon's rank sum test). Comparison of the results obtained directly after 14 days of reflux and two, four and six weeks after Roux-en-Y diversion (Fig. 5.19) showed no significant differences among these groups.

Comparison of the results obtained directly after 28 days of reflux and two, four and six weeks after Roux-en-Y diversion (Fig. 5.20) showed significant differences between the samples collected two and six weeks after Roux-en-Y diversion ($p < 0.05$), and between the samples collected four and six weeks

Table 5.VIII. COLLAGEN CONTENTS OF GROUPS GBP + BP COMBINED

	$\frac{\text{dry fat free tissue (mg)}}{\text{wet tissue (mg)}} \times 100$				% collagen of dry fat free tissue			
Group	n	$\bar{x}$	Range	p	n	$\bar{x}$	Range	p
Control	9	19.9	17.8-22.7		9	14.5	11.3-17.1	
7.C	8	16.6	14.7-18.8	<0.01	6	15.4	11.2-18.3	ns
7.2	7	18.5	8.3-22.5	ns	7	13.4	10.8-16.7	ns
7.4	9	18.5	12.0-22.1	ns	8	14.1	7.3-22.2	ns
7.6	10	19.2	15.9-22.4	ns	9	18.7	12.9-24.5	<0.05
14.C	6	15.3	14.3-16.5	<0.01	6	14.6	10.9-20.3	ns
14.2	8	17.1	6.7-20.0	ns	6	13.3	8.7-16.1	ns
14.4	11	19.4	16.4-23.3	ns	8	16.2	11.7-20.1	ns
14.6	7	19.8	17.9-22.0	ns	7	18.5	6.1-22.5	ns
28.C	10	15.6	11.9-20.9	<0.01	10	17.4	11.0-28.0	ns
28.2	7	16.5	9.1-19.4	<0.01	6	14.0	8.5-22.6	ns
28.4	9	19.3	17.6-20.6	ns	8	17.7	10.3-22.7	ns
28.6	10	20.0	16.2-23.7	ns	4	23.0	21.5-23.9	<0.01
42.C	9	14.2	7.2-16.7	<0.01	9	17.7	11.8-22.8	<0.05
42.2	8	18.9	16.4-22.1	ns	8	15.1	7.3-20.9	ns
42.4	10	18.7	15.9-20.6	ns	7	18.2	11.7-22.9	<0.05
42.6	10	19.5	17.0-21.8	ns	5	22.9	19.6-28.2	<0.01

The p values indicate a significant difference between experimental values and control values obtained from non-operated rats (Wilcoxon's rank sum test).

after Roux-en-Y diversion ($p < 0.02$). Comparison of the results obtained directly after 42 days of reflux and two, four and six weeks after Roux-en-Y diversion (Fig. 5.21) showed significant differences between samples obtained directly after 42 days of reflux and those obtained after six weeks of Roux-en-Y diversion ($p < 0.02$), and between samples obtained two and six weeks after Roux-en-Y diversion ($p < 0.05$).

In summary, the results of the collagen content determinations indicate an early increase of collagen in the oesophageal wall after a short period of reflux,

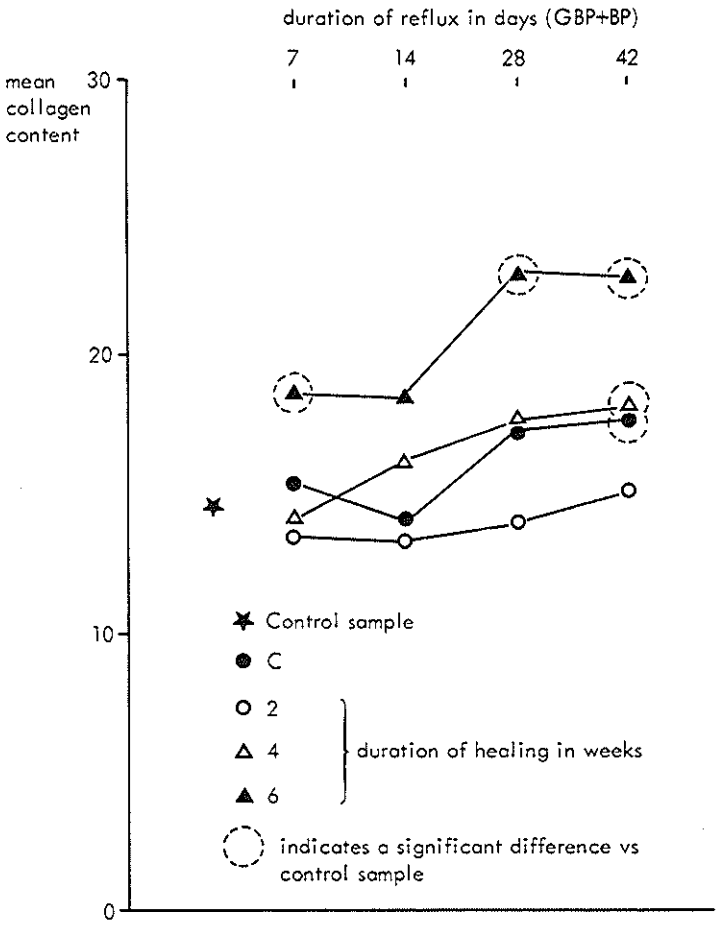


Fig. 5.16. Combined (GBP + BP) results of the collagen content determinations. Control sample indicates the mean value of the collagen content determinations obtained from 9 non-operated rats.

which becomes more evident with a longer duration of reflux, reaching statistically significant levels after 42 days of reflux. Furthermore, the fibrotic changes induced by the reflux oesophagitis become more evident with an increasing time interval between the end of the reflux period and the taking of the oesophageal wall sample.

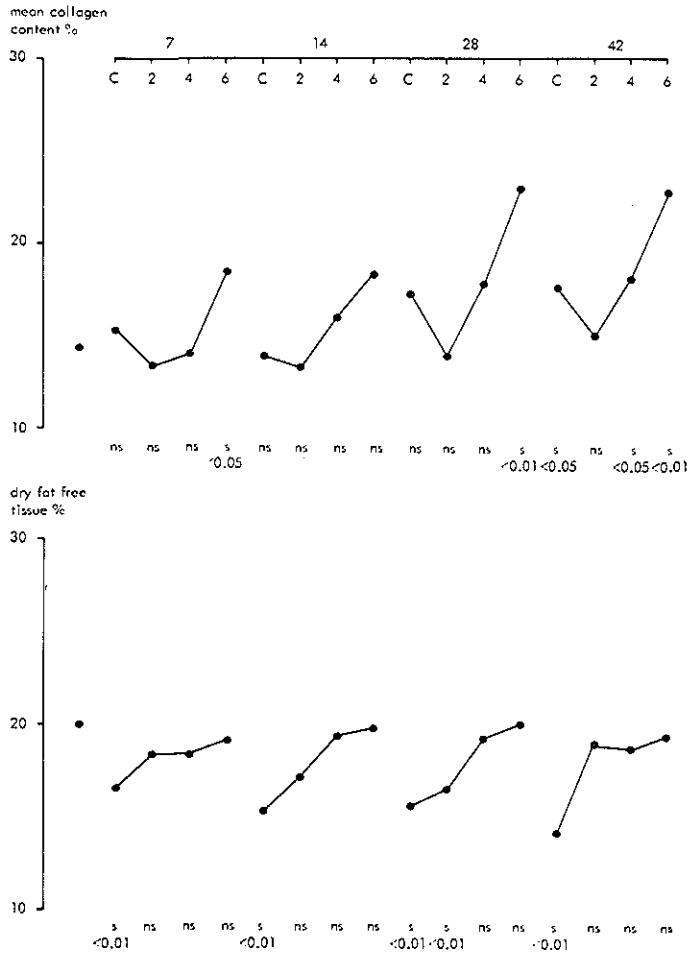
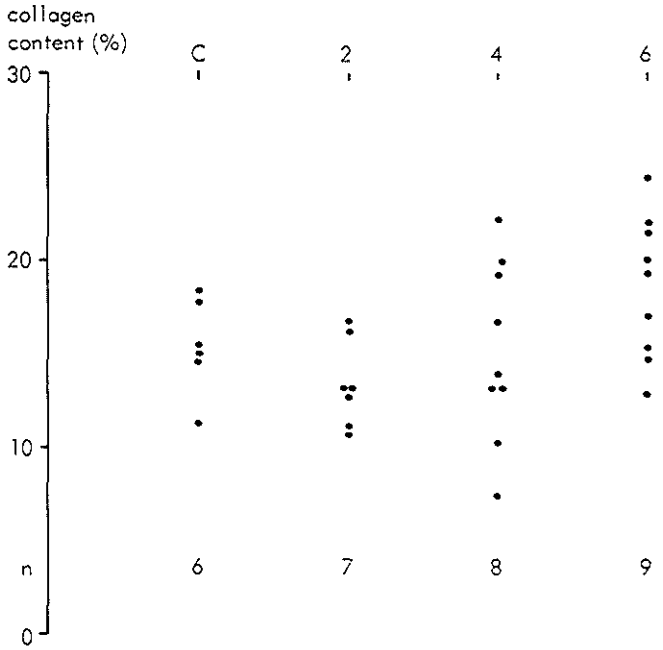


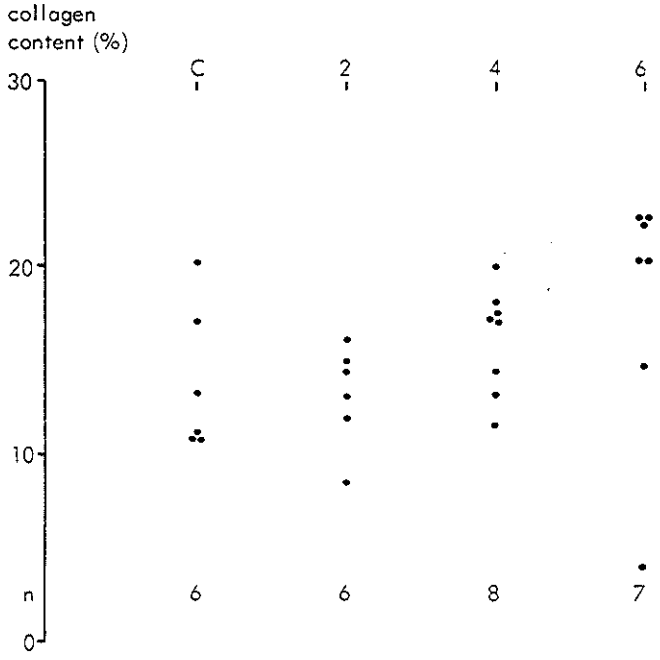
Fig. 5.17. Combined (GBP + BP) results of the collagen content determinations and corresponding DFFT percentages. p values indicate significance level versus control values obtained from non-operated rats.



nullhypothesis: A ≠ B

A	B		critical region	p
7.C vs	7.2	52	27 - 57	ns
	7.4	50	29 - 61	ns
	7.6	34	31 - 65	ns
7.2 vs	7.4	51	38 - 74	ns
	7.6	36	37 - 82	<0.02
7.4 vs	7.6	54	45 - 99	ns

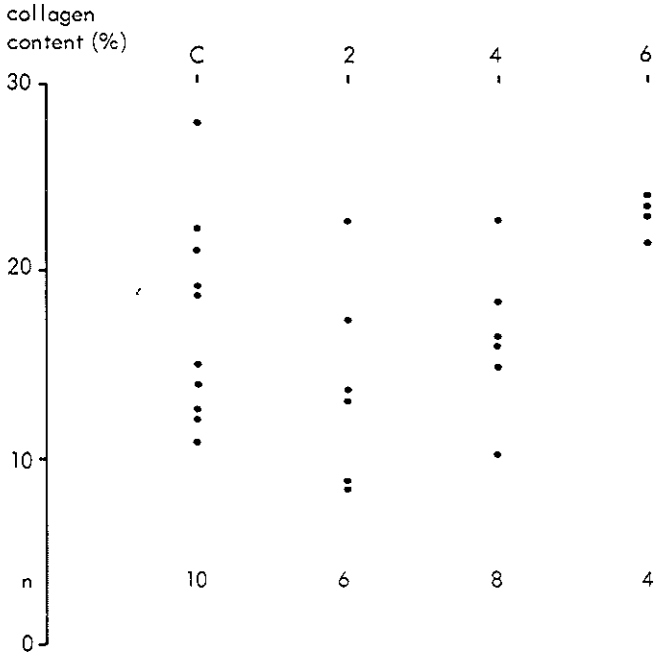
Fig. 5.18. Collagen content after 7 days of reflux and the influence of healing (GBP + BP).



nullhypothesis: A ≠ B

A	B	T	critical region	p
14.C vs	14.2	39	26 - 52	ns
	14.4	34	29 - 61	ns
	14.6	29	27 - 57	ns
14.2 vs	14.4	31	29 - 61	ns
	14.6	29	27 - 57	ns
14.4 vs	14.6	49	46 - 82	ns

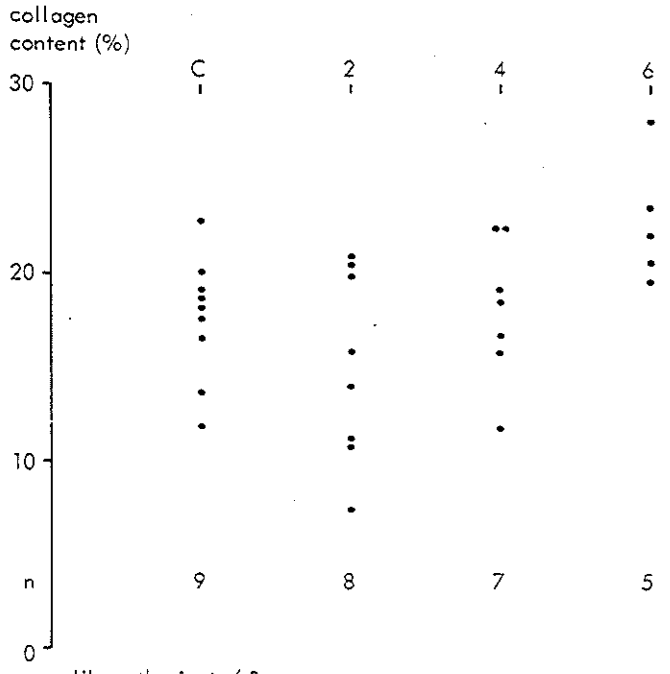
Fig. 5.19. Collagen content after 14 days of reflux and the influence of healing (GBP + BP).



nullhypothesis: A ≠ B

A	B	T	critical region	p
28.C vs	28.2	95	66 - 104	ns
	28.4	89	72 - 118	ns
	28.6	60	60 - 90	=0.05
28.2 vs	28.4	34	29 - 61	ns
	28.6	22	23 - 43	<0.05
28.4 vs	28.6	37	40 - 64	<0.02

Fig. 5.20. Collagen content after 28 days of reflux and the influence of healing (GBP + BP).



nullhypothesis: A ≠ B

A	B	T	critical region	p
42.C vs	42.2	89	60 - 102	ns
	42.4	75.5	57 - 96	ns
	42.6	49	50 - 85	<0.02
42.2 vs	42.4	54	46 - 82	ns
	42.6	40	42 - 70	<0.05
42.4 vs	42.6	33.5	33 - 58	ns

Fig. 5.21. Collagen content after 42 days of reflux and the influence of healing (GBP + BP).

5.4. DISCUSSION

In this study an operation which induced pancreatic juice reflux into the oesophagus of the rat uniformly resulted in the development of a panmural oesophagitis as was previously reported by Levrat (1961), Kranendonk (1980), and in Chapter 4 of this thesis. The first histological description of this induced oesophagitis was given by Levrat and co-workers (1961), who interpreted the changes in the oesophagus as a combination of three histological processes, namely epithelial hyperplasia with major hyperkeratosis, ulceration of the mucous membrane not entering deeper than the submucous membrane, and diffuse inflammatory infiltration of the mucous and submucous layers. Kranendonk (1980) described the time sequence of histological changes. Among his findings were the following: within seven days after the induction of reflux, a definite increase of connective tissue was notable, which became more marked when the duration of reflux had been longer. After 42 days of reflux he observed loss of elasticity and shortening of the oesophagus and ascribed this to "the progressive extensive fibrosis throughout the layers of the oesophageal wall".

In the present study also an obvious tendency of the oesophageal lesions to become more extensive with a longer duration of reflux was noted. Ulcerative lesions were accompanied by a panmural infiltrate in which the pre-existing structures could not even be recognized. Kranendonk has suggested in his thesis (1980) that this finding should be interpreted as evidence of the destruction of the pre-existing structures due to the oesophagitis. However, the results of the present study have demonstrated that once the oesophagitis has healed, a complete recovery of these pre-existing anatomical structures occurred. It is therefore unlikely that those structures could have been totally destructed during the preceding oesophagitis period. The inflammatory changes must have acted as a screen obscuring the normal anatomic structure of the oesophageal wall.

The results of the collagen content determinations show that reflux oesophagitis in the rat is associated with an increase of the collagen content of the oesophageal wall, which is already evident after seven days of reflux. This increase progresses when the period of reflux is extended and parallels the development of more serious inflammatory changes in the wall of the oesophagus. The increase proceeds to significant differences between the experimental groups and non-operated control rats after forty-two days of reflux.

When the reflux of pancreatic juice containing intestinal contents into the oesophagus was abolished by a Roux-en-Y diversion, this resulted in healing of the oesophagitis. Induction and abolition of pancreatic juice reflux into

the oesophagus could be adequately monitored by trypsin determinations in the oesophageal contents before and after these procedures. Already fourteen days after the reflux-abolishing operation only intact epithelium was found, though hyperplasia and hyperkeratosis were still frequently encountered. Thus the epithelial layer might have contributed to a larger proportion than normal to the weight of the oesophageal samples. This could account for the relatively low collagen content of oesophageal wall samples collected at this time in the experiment. With longer duration of healing the epithelial layer returns to normal and inflammatory changes disappear. This is accompanied by a distinct rise in collagen content leading to significant differences in several experimental groups. Collagen content was expressed as a percentage of dry fat free tissue weight. We suggest that the changes in collagen content described reflect changes in the relative contributions of the different tissues in the sample to the total dry fat free tissue weight. Studies of the healing of colonic anastomoses in rats (Cronin et al., 1968) have shown that in the initial phase of healing there is both a breakdown of old and a synthesis of new collagen with a negative net result. However, breakdown ceases after the fifth day and from that moment on a gradual rise in collagen content occurs to a level just below control levels around the 14th day. These changes in collagen content are found as far as 2.5 cm away from the anastomosis. Whether these findings also apply to an oesophagojejunostomy is not known. If so, then they might have influenced collagen levels in the experimental groups studied after seven and fourteen days of reflux.

The anaemia that was encountered in all rats might be related to blood loss from the inflamed oesophagus. However, healing of oesophagitis was not accompanied by improved haematocrit levels. In rats, absorption of iron and vitamine B_{12} are impaired after total gastrectomy (Bussabarger, 1936; Watson and Fleney, 1955) and the exclusion of the stomach from the food passage may have had the same effects.

In this experiment reflux oesophagitis was found to be associated with a definite shortening of the oesophagus, thus confirming the findings of previous authors. Helsingen (1961) postulated that the process of shortening was initiated by a muscular contracture of the oesophagus. Kranendonk (1980) emphasized the role of the progressive fibrosis he noted. The healing of oesophagitis in the present study was associated with a gradual and probably partial recovery of the oesophageal length in all experimental groups indicating that, although a certain influence of fibrosis on the oesophageal length is possible, it is certainly not the only factor determining the degree of shortening.

5.5. SUMMARY AND CONCLUSIONS

The effect of healing of oesophagitis on the oesophageal wall is not well known. The aetiology of its most serious complication, namely fibrous stricture, is obscure. In rats the surgical induction of reflux of pancreatic juice results in a panmural oesophagitis. Fibrous strictures in man are usually also accompanied by panmural oesophagitis. Therefore, an investigation was done with the objective to study, in surgically induced reflux oesophagitis in rats, the effects of the duration of reflux and of healing on the collagen content of the oesophageal wall. The induction of reflux oesophagitis was reflected in weightloss and anaemia. After a longer period of reflux a shorter, oedematous oesophagus was found.

The results of the study show that reflux oesophagitis in rats can be cured by a duodenal diversion procedure creating a Roux-loop of $\pm$ 25 cm. The induction and abolition of the reflux of pancreatic juice can be adequately monitored by active trypsin determinations in oesophageal washout samples. The healing of the oesophagitis was accompanied by weightgain, but not by correction of the anaemia. The healing oesophagus regained length and lost oedema.

The collagen content of the oesophageal wall increased with longer duration of the reflux. After 42 days of reflux, collagen content was significantly higher than that of non-operated rats ($p < 0.05$), although strictures were not encountered. After abolition of the reflux collagen contents increased with a longer duration of healing, leading to significant differences with the collagen contents of non-operated rats six weeks after three out of four reflux periods studied. Microscopic quantification of the mural fibrosis proved too inaccurate to confirm the findings of the collagen content determinations.

CHAPTER 6: GENERAL DISCUSSION AND CONCLUSIONS

6.1. THE AETIOLOGY OF REFLUX OESOPHAGITIS

There is still considerable controversy as to the nature of the offending agent involved in the aetiology of reflux oesophagitis in man. Active components from both gastric juice and duodenal contents are thought to be potentially harmful to the oesophageal mucosa. Animal experiments, using different species in either perfusion studies, in which the oesophagus is continuously exposed to a known substance, or studies in which reflux of a certain part of the gastro-intestinal secretions into the oesophagus is induced by surgical means, have been used extensively to elucidate the damaging potential of the various upper gastro-intestinal secretions.

In rats, an elegant experiment was performed by Kranendonk (1980), who used a model in which reflux of gastric juice, bile, and pancreatic juice into the oesophagus had been made possible by different operative procedures. The effects of the possible reflux into the oesophagus of these juices alone, or in combination, could thus be studied. Kranendonk concluded that reflux of pancreatic juice alone, or in combination, resulted in severe panmural oesophagitis, whilst reflux of neither gastric juice nor bile caused any damage to the oesophageal mucosa at all. The question remaining after this study was whether the substances that were thought to reflux into the oesophagus actually did show up in the oesophageal contents. Therefore, a similar experiment was undertaken in rats with the objective to examine the oesophageal contents for evidence of the reflux that was supposed to have been induced. Active trypsin was found in high concentrations in the oesophagus of rats in which pancreatic juice reflux was thought to be induced, be it alone, or in combination with bile or gastric juice, and this was associated with a 100% incidence of panmural oesophagitis.

Furthermore, high bile acid concentrations could be detected in the oesophageal contents when bile reflux had been induced, but their detection had no correlation with the occurrence of oesophagitis.

Active pepsin could never be demonstrated in the oesophageal contents. The secretory function of the stomach after a reflux-inducing operation in the rat was therefore studied with the Hollander-test, which demonstrated an almost complete absence of acid production from the stomach after a reflux-inducing operation. It was concluded that during this operation an inadvertent vagotomy had been performed.

Therefore, the results of our experiments support the concept of Kranendonk (1980) that reflux of pancreatic juice into the rat oesophagus is associated with the development of a panmural oesophagitis. Our results also demonstrate that conclusions about the damaging effect of any substance should only be drawn when it has been shown that this very substance actually reaches the area it is supposed to damage.

Other authors have drawn attention to a possible role for pancreatic juice in the aetiology of reflux oesophagitis. Cross and Wangensteen (1951) surgically induced reflux of pancreatic juice into the oesophagus of dogs, which resulted in oesophagitis. Rat experiments by Lambert (1962) confirmed these findings, and Lambert could also partially prevent the development of oesophagitis in reflux of pancreatic juice by the daily intraperitoneal administration of a trypsin inhibitor, thus indicating trypsin as a major factor in the development of reflux oesophagitis in rats.
In vitro testing of rabbit oesophageal mucosa with a trypsin solution demonstrated a distinct damaging effect of trypsin at a pH of 7.4 (Kivilaakso, 1980). Bateson et al. (1981) incubated human oesophageal mucosa biopsies in a solution of both trypsin and lipase at a neutral pH. Examination of these exposed biopsies by electron microscopy demonstrated changes comparable with those seen in oesophagitis.

Our finding that high bile acid concentrations in the rat oesophagus are not correlated with the development of oesophagitis is opposed by other reports in the literature. Cross and Wangensteen (1951) and Moffat (1965) in dogs, and Gillison (1972) in monkeys, surgically induced bile reflux into the oesophagus and saw the development of oesophagitis. None of them, however, tested the composition of the refluxting juice as it reached the oesophagus, and from the description of the experimental model used by these authors, it is obvious that also other corrosive agents like pancreatic enzymes could have become part of the refluent bolus. Their conclusions about the effect of bile on the oesophageal mucosa therefore seem to be based on assumptions rather than on solid evidence.

It is assumed that increased permeability to hydrogen ions is an early sign of oesophageal mucosal injury (Kivilaakso, 1980), similar to gastric mucosal injury (Ritchie, 1977). Safaie-Shirazi et al. (1975) measured a five-fold increase of the permeability of the human oesophagus to hydrogen ions after exposure to bile salts in the presence of acid. They also demonstrated this phenomenon in dogs. Chung et al. (1974) made similar observations in an in vivo study in rabbits. Kivilaakso (1980) studied rabbit oesophageal mucosa in vitro, and measured an increased permeability to

hydrogen ions after exposure of the mucosa to a solution of conjugated bile salts at a pH of 3.5. When deconjugated bile salts were used, a similar effect was noted at a pH of 7.4. The findings of Kivilaakso have been confirmed by Harmon et al. (1981) in an in vivo study in rabbits.

It could well be that the lack of free hydrogen ions during bile reflux in our experiment explains the absence of evidence of oesophageal damage in that case.

Admixture of bile with pancreatic juice leads to the hydrolization of lecithin from bile by phospholipase A from pancreatic juice to lysolecithin, which has a potential damaging effect on gastric mucosa (Kivilaakso, 1976), but only a moderate effect on oesophageal mucosa as demonstrated by an in vitro study on rabbit oesophageal mucosa (Kivilaakso, 1980). In our study lysolecithin reflux was not monitored, but the addition of bile to pancreatic juice reflux did not result in a more extensive oesophagitis. The role of lysolecithin in the aetiology of reflux oesophagitis remains to be established.

Results obtained from an experimental study in animals derive at least part of their importance from their relevance for the human situation. In human reflux oesophagitis an increased gastro-oesophageal reflux (DeMeester et al., 1976), due to frequent inadvertent transient relaxations of the lower oesophageal sphincter (Hauser et al., 1979; Dent, 1980), together with a delayed clearance of the refluent gastric bolus from the oesophagus (Little et al., 1980), leads to prolonged exposure of the oesophageal mucosa to harmful elements in the gastric contents. The composition of the gastric contents is determined by gastric secretion (acid and pepsin) and regurgitation of duodenal contents into the stomach. This so-called duodenogastric reflux has been shown to be increased in patients with symptoms of reflux oesophagitis (Kaye and Showalter, 1974; Crumplin et al., 1974; Stol et al., 1974) and is thought to be caused by pyloric dysfunction, of which also the delayed gastric emptying that is seen in reflux oesophagitis patients (Little et al., 1980; Donovan et al., 1977) is a sequel. Pancreatic juice reflux, contrary to biliary reflux, has not received much attention by other authors, since it was thought (Gillison, 1972) that pancreatic enzymes were rapidly destroyed in the stomach. However, Wenger (1971) has shown the prolonged presence of trypsin in the stomach after a testmeal at a pH between 3.5 and 7, a pH at which trypsin is stable and partially active. Furthermore, Kranendonk et al. (1980) demonstrated the ubiquitous presence of active trypsin in the stomach of patients with symptoms of oesophagitis with a distinct relation between a high level of active trypsin in the stomach, and the finding of severe ulcerative oesophagitis.

It is therefore postulated that active trypsin, the presence of which in the oesophagus of rats is associated with severe oesophagitis, and other enzymes from pancreatic juice could play an important role in the development of reflux oesophagitis in man.

6.2. THE CONSEQUENCES OF REFLUX OESOPHAGITIS

The induction of reflux oesophagitis in rats and the histological consequences thereof have been described by Levrat (1961) and Kranendonk (1980). Kranendonk emphasized the progressive fibrosis in, and the possible destruction of pre-existing structures of the oesophageal wall. We therefore again studied the histological consequences of reflux oesophagitis in rats, and tried to biochemically measure the supposed fibrosis by determination of the collagen content of oesophageal wall specimens. Furthermore, healing of the oesophagitis was induced by eliminating the reflux of corrosive agents, and the consequences of healing were monitored.

The results of this experiment are described in Chapter 5, and show that reflux oesophagitis in rats is accompanied by weightloss, which progresses with longer duration of the disease. The weight loss is reversible after elimination of the reflux. An also progressive, but irreversible, anaemia was obvious. The most important observation was a progressive fibrosis of the oesophageal wall worsening with a longer duration of the reflux. Six weeks after elimination of the reflux, the fibrosis seemed to have progressed even more, with a similar trend in all 4 groups studied at that time, leading to significantly higher collagen contents in 3 of these groups when compared to control values of non-operated rats.

The ability of the rat oesophagus to recover from a serious panmural oesophagitis once the reflux of the corrosive agent has been abolished is striking. Microscopic studies proved adequate in monitoring this process of healing, but were too inaccurate, even when specific collagen stains were used, to provide a visual reflection of the biochemically measured changes in the collagen contents.

The response of reflux oesophagitis in man to surgical or medical treatment is usually monitored clinically and endoscopically. There is very little information about the histological features of healing of oesophagitis. A recent study by Wesdorp (1978) concerning the effect of cimetidine treatment in reflux oesophagitis describes an improvement in histological grading of the oesophagitis, but no details are given. Brand (1979), when reviewing, among other things, the histology of the oesophagus before and

after anti-reflux surgery refers to the criteria of Ismael-Beigi et al. (1970) when reporting an improvement of the histological picture. There is also very little information about the time sequence of histological inflammatory changes that are caused by gastro-oesophageal reflux.

However, the development of the most serious complication, being a stricture, seems to be related to long-standing disease (Ahtaridis et al., 1979), although strictures are known to have occurred after a short period of symptomatic disease, or after the oesophagitis had healed.

It seems possible that in humane oesophagitis a progressive fibrosis of the oesophageal wall can be a sequel of long-standing reflux disease, comparable to our observations in oesophagitis in rats. A stricture could be the end-result of such a fibrosis. If the development or progression of fibrosis in humane reflux oesophagitis is to be monitored, microscopic evaluation could prove to be inadequate, and instead a biochemical determination of the collagen content of oesophageal mucosal biopsies should be performed. Monitoring the collagen content in these biopsies could be of help in detecting patients at risk for the development of a stricture.

6.3. OESOPHAGEAL MURAL FIBROSIS AND TRYPSIN

The main problem with stricture formation in the oesophagus due to reflux oesophagitis is that it is not known what, if any, circumstances predispose to the development of mural fibrosis and subsequent stricture formation. A fibrous stricture in the oesophagus is usually associated with panmural oesophagitis in which there is increased mural fibrosis, and which is macroscopically characterized by shallow ulcerative lesions (Postlethwait, 1979). This ulcerative type of oesophagitis was in the study by Kranendonk et al. (1980) associated with high levels of active trypsin in the stomach. The panmural ulcerative oesophagitis in rats that has been described in Chapters 4 and 5 of this thesis was correlated with the presence of active trypsin in the oesophageal contents. Active trypsin is also known to stimulate the proliferation of humane fibroblasts in tissue culture (Pohjanpelto, 1977).

It is therefore postulated that there is a relation between the presence of active trypsin in the refluent juice and the development of mural fibrosis in the oesophageal wall.

SUMMARY

The nature of the damaging agent coming from the digestive juices which is responsible for the development of reflux oesophagitis is subject to much discussion. In rats, some studies suggest a damaging effect of pancreatic juice, while other studies indicate bile or gastric juice as the main corrosive factor.

In this thesis, the influence of various digestive juices on the oesophagus of rats was investigated more closely. Besides, the effects of oesophagitis on the composition of the oesophageal wall with respect to the collagen content were studied. Further investigations were concerned with the recovering potential of the rat oesophagus after the corrosive factor had been eliminated.

In Chapter 1 a review of the literature concerning the aetiology of reflux oesophagitis is given, after which the objectives of the study are being formulated.

In Chapter 2 information about experimental animals and techniques is presented. The statistical analysis of the results is discussed.

In Chapter 3 the effects of reflux of gastric juice into the oesophagus are described, as well as the effects of a reflux inducing operation on the acid production of the stomach. It is shown that reflux of gastric juice into the oesophagus does not result in the appearance of active pepsin in the oesophageal contents, nor in the development of oesophagitis. After a reflux inducing operation the acid production of the stomach is shown to be diminished as if a truncal vagotomy had been performed.

In Chapter 4 it is shown that operative induction of reflux of pancreatic juice into the oesophagus results in the appearance of active trypsin in the oesophageal contents as well as in oesophagitis. Reflux of bile is demonstrable by the determination of bile acid levels in the oesophageal contents, and does not influence the development of oesophagitis.

In Chapter 5 a description is given of the determination of the collagen content of the oesophageal wall after a reflux inducing operation, with which a fibrosis of the oesophageal wall is demonstrated that increases with longer duration of the reflux. After elimination of the reflux by a Roux-en-Y transformation of the anastomosis healing of the oesophagitis is observed with continuing and possibly even increasing fibrosis.

In Chapter 6 the results of the experiments described in this thesis are being discussed in relation to the existing literature.

SAMENVATTING

De aard van het schadelijke agens afkomstig uit de spijsverteringssappen dat verantwoordelijk is voor het ontstaan van refluxoesophagitis is onderwerp van veel discussie. Sommige rattenstudies suggereren een beschadigende werking van pancreassap, terwijl andere juist gal of maagsap als de belangrijkste beschadigende factor aanwijzen.

In dit proefschrift werd de invloed van verschillende spijsverteringssappen op de ratteoesophagus nader bestudeerd. Bovendien werd onderzocht wat de gevolgen zijn van oesophagitis voor de samenstelling van de slokdarmwand, met name wat betreft het bindweefselgehalte. Verder werd gekeken naar het herstelvermogen van de ratteoesophagus, nadat de beschadigende factor was geëlimineerd.

In hoofdstuk 1 wordt aan de hand van een literatuuroverzicht de aetiologie van refluxoesophagitis besproken en de doelstellingen van het onderzoek geformuleerd.

In hoofdstuk 2 worden algemene gegevens betreffende de proefdieren en de proefopzet vermeld. Ook wordt de statistische bewerking van de resultaten besproken.

In hoofdstuk 3 worden de gevolgen van maagsapreflux in de oesophagus beschreven, alsmede de gevolgen van een reflux-inducerende operatie voor de zuurproduktie van de maag. Aangetoond wordt dat maagsapreflux in de oesophagus niet leidt tot het verschijnen van actieve pepsine in de oesophagusinhoud en dat er ook geen oesophagitis ontstaat. Na een reflux-inducerende operatie blijkt de zuurproduktie van de maag verminderd te zijn als na een stamvagotomie.

In hoofdstuk 4 wordt aangetoond dat operatief geïnduceerde reflux van pancreassap in de oesophagus leidt tot het verschijnen van actieve trypsine in de slokdarminhoud en tot oesophagitis. Reflux van gal is aantoonbaar door het meten van galzure zouten in de slokdarminhoud en heeft geen invloed op het al dan niet ontstaan van oesophagitis.

In hoofdstuk 5 wordt beschreven hoe na operatief geïnduceerde refluxoesophagitis het bindweefselgehalte van de slokdarm wordt bepaald, waarmee een fibrosering wordt aangetoond die toeneemt bij een langere duur van de reflux. Na eliminatie van de reflux door een Roux-en-Y omzetting van de anastomose geneest de oesophagitis, maar de fibrosering neemt eerder toe dan af.

In hoofdstuk 6 worden de resultaten van het eigen onderzoek besproken en gerelateerd aan de bestaande literatuur.

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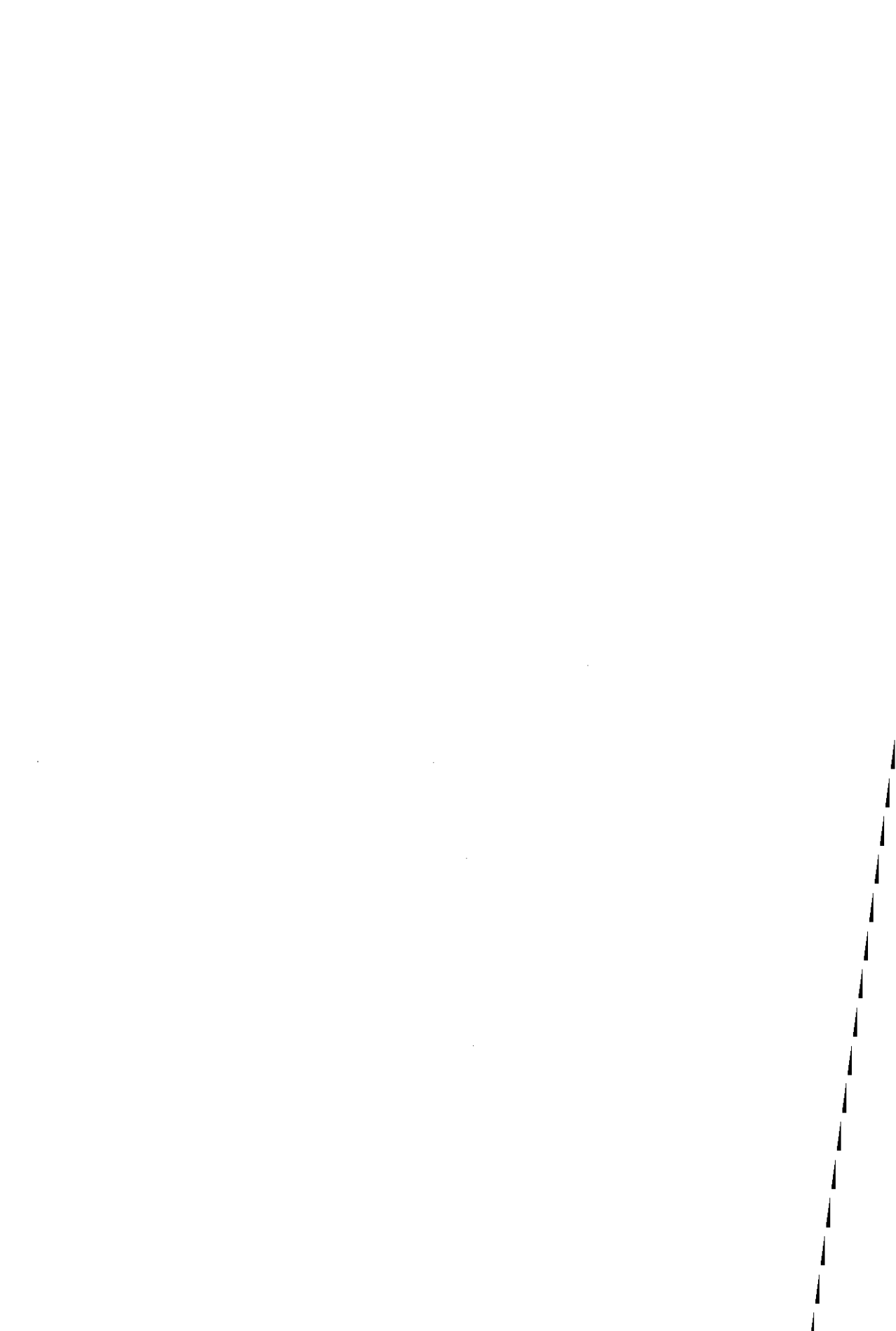

CURRICULUM VITAE

De auteur van dit proefschrift werd in 1947 te Utrecht geboren. Hij behaalde het eindexamen H.B.S.-B aan de Rijks-H.B.S. te Heerenveen in 1964. In hetzelfde jaar begon hij met de medische studie aan de Rijksuniversiteit in Utrecht. In 1971 legde hij het artsexamen af.
Hierna bereidde hij zich voor op een werkkring in de tropen. De eerste chirurgische ervaringen werden opgedaan bij dr. A.P. Brinkhorst in het Ikazia Ziekenhuis te Rotterdam, alwaar ook de gynaecologie en verloskunde voor de eerste maal aan bod kwamen (dr. H.F. Heins). Hierna volgde de Nationale Tropencursus voor Artsen en uitzending naar Malawi waar hij van 1973 tot 1975 werkte als Government Medical Officer, eerst in Lilongwe en later in Mzimba.
Na terugkeer uit de tropen volgde nog een periode chirurgische scholing bij dr. A.P. Brinkhorst. Van juni 1976 tot maart 1978 was hij werkzaam op de afdeling Gynaecologie en Verloskunde van het Academisch Ziekenhuis in Utrecht (Hoofd: prof.dr. A.A. Haspels). Hierna begon hij met de opleiding tot algemeen chirurg in het Academisch Ziekenhuis Dijkzigt te Rotterdam onder leiding van prof.dr. H. van Houten en prof.dr. J. Jeekel.

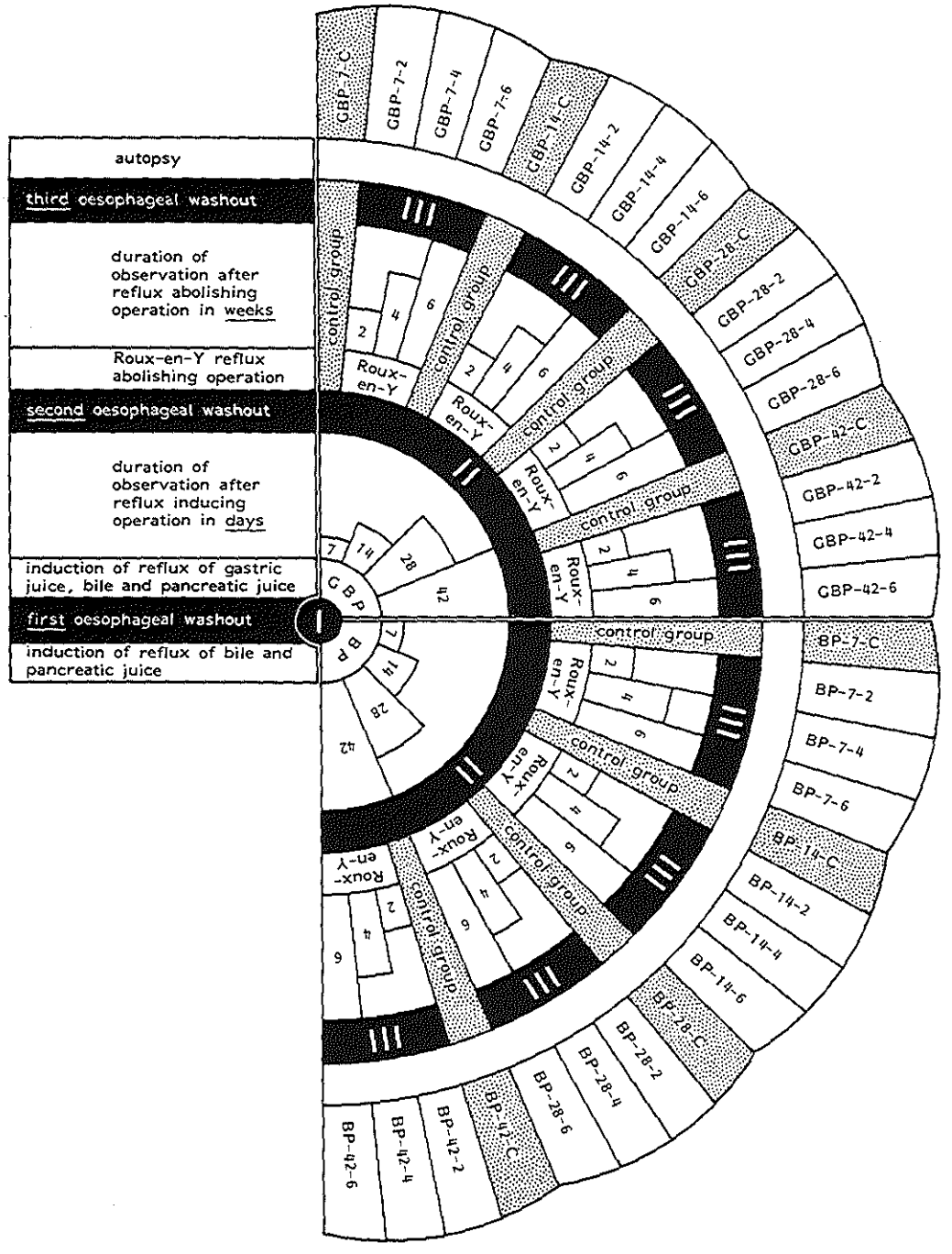

Fig. 5.1. General design of the experiment.