A STUDY OF EXPERIMENTAL CASTRIC ULCERS AND PHLEGMOMES

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SUMMARY

Various models for an experimental study of gastric ulcers and erosions were sought and high incidences of erosion or ulceration were obtained using stress (restraint), anti-inflammatory drugs (phenylbutazone, aspirin), a corticosteroid (prednisolone) and physical or chemical trauma (electrocautery or acetic acid). Reproducible results were obtained only by carefully controlling variables such as sex, bodyweight, temperature and season.

Histochemically, erosions and ulcers were associated with a loss of mucus, particularly from the regenerative zone of the mucosa. Biochemical studies after erosion formation confirmed the decrease in mucus content and also showed an altered carbohydrate composition of the mucus.

Studies of mucous glycoprotein synthesis by measuring the rate of incorporation of labelled carbohydrate and amino acid suggested that one mode of action of stress and ulcerogenic drugs may be inhibition of the glycosylation of the mucus glycoprotein and hence a decreased synthesis of glycoprotein or the synthesis of a modified glycoprotein or both.

Prostaglandins \( \text{P}_1 \), \( \text{P}_2 \) and \( \text{P}_4 \) were found to decrease the incorporation of carbohydrate and amino acid. This effect may be produced by stimulation of adenyl cyclase and elevation of cyclic AMP levels as the dibutyryl analogue of cyclic AMP and a phosphodiesterase inhibitor, theophylline, were found to have similar effects. The dibutyryl analogue of cyclic GMP was found to have no effect on glycoprotein synthesis. Glycaemia was also found to be important as hypoglycaemia decreased glycoprotein synthesis and increased the susceptibility to erosion formation. It appears that a constant supply of glucose to the mucosal cells is necessary for synthesis of the mucus glycoprotein and normal cell function.

The drug carbenoxolone sodium and an analogue ciclooxolone sodium were shown to be effective against stress-induced erosions and also promoted the healing of electrocautery ulcers. The
mechanism of action may be allied with increased mucus synthesis as both drugs were shown to increase the glycosylation of mucus glycoproteins.
To

Anna

and to

my Father

"That I for poor auld Scotland's sake
Some useful plan or book could make"

Robert Burns
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CHAPTER 1

"PEPTIC ULCER — A REVIEW"

1.1 Introduction
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1.3 Modern aspects of peptic ulcer
1.1 Introduction

Peptic ulcer disease is a common enough problem both to the patient and to society. Intensive research has been carried out in this area for a number of years but the cause of the disease is still largely unknown in spite of greater knowledge of the conditions necessary for peptic ulcers to form. Probably a major difficulty lies in the fact that 'peptic ulcer' is a broad description for a number of lesions and the factors responsible may vary according to the type of ulcer.

Menguy (1970) gave the following classification of peptic ulcer:

1) Duodenal ulcer
   Prepyloric ulcer
   Combined gastric and duodenal ulcer

2) Gastric ulcer

3) Acute gastric mucosal erosions

Johnson (1965) defined 3 groups in gastric ulcer: Type 1 were single ulcers on the lesser curve associated with hyposecretion of hydrochloric acid: Type 2 were gastric ulcers associated with duodenal ulcers: Type 3 were prepyloric ulcers. Types 2 and 3 were associated with the hypersecretion of hydrochloric acid. Recent evidence suggests that there may also be two separate populations of patients with duodenal ulcer (Lam & Sircus, 1975).

The preoccupation with acid and pepsin secretion over the years may be partly responsible for the lack of progress with the understanding and treatment of the disease but in recent years more attention has been paid to tissue resistance and the factors which protect the normal mucosa against acid-pepsin digestion. Peptic ulcer is now being seen as the outcome of an imbalance of the aggressive forces of acid, pepsin, biliary reflux, ingested irritants (including drugs) and pancreatic enzymes and the defensive forces, including mucosal blood supply, mucus and cell proliferation (Avery
Jones, 1975). This sort of approach has contributed greatly to our knowledge of the disease and in fact the major therapeutic advance has been mediated by a drug which strengthens the defensive forces rather than reducing the aggressive ones.

1.2. Historical aspects

Although the first description of chronic gastric ulcer is frequently attributed to Cruveilhier (1829) descriptions of peptic ulcer symptoms are to be found long before this time. Goldstein (1939) claimed that the history of gastric and duodenal ulcer went back to neolithic days (3000 - 1500 B.C.). No doubt the earliest description of gastric ulcer surgery is to be found on the pillars of the temple of Aesculapius at Epidaurus where an inscription dating from around 4 B.C. tells of the god Asklepios opening the stomach of a man with an ulcer, cutting out the ulcer and sewing him up again. Physicians in ancient times were concerned also with the treatment of peptic ulcers. A bland diet and neutralising drugs were recommended as early as the first century A.D. by Celsus (Goldstein, 1939).

Even before Cruveilhier's description of peptic ulcer, the resistance of the normal stomach to digestion was a subject of interest to experimental physiologists. John Hunter (1772) attributed the power of the mucosa to resist destruction to "an unknown living principle". No further ideas on the subject were forthcoming until 1856 when Bernard and Pavy demonstrated that living tissues were digested when taken into the stomach. These initial studies were the starting point from which more definite theories of the resistance of the living stomach to digestion began to take form.

Bernard (1856) and Pavy (1853, 1863) both failed to consider that acid in the gastric juice was a critical factor. Frenzel (1886-7) showed that digestion of living tissue required both acid and pepsin but Matthes (1893) considered that acid alone was partly responsible for the destruction of living
tissue. Dragstedt (1935) has shown that there is a critical concentration of HCl required below which digestion does not take place. Up to the present day there is still controversy over the role of acid and pepsin in the aetiology of peptic ulcer.

Bechamp (1882) recognised that the reason that the gastric mucosa was not destroyed by acid and pepsin was that there is constant formation of new cells to take the place of those disappearing through usage. The significance of the continual renewal of the surface epithelium has virtually been ignored until modern times (Section 1.3.9).

In his theory of resistance of digestion of the stomach, Bernard (1856) also included the protective action of mucus although no experiments were carried out to prove his point. Harley (1860) believed that the mucous secretion of the cells was the most important factor in the protection of the stomach. He showed that if a stomach is divided into two parts and the mucus removed from one of the parts then this part was rapidly destroyed by gastric juice while the part retaining its mucus was unaffected. Bucher (1932) showed that gastric mucus delayed the diffusion of acid and pepsin while de Klug (1907) found that it inhibited peptic activity, an observation confirmed by others (Babkin & Komarov, 1932; Komarov, 1935). Although some authors reported that gastric mucus is resistant to acid digestion (e.g. Pekelharing, 1902) it is known to be degraded by acid (Bucher, 1932). The buffering capacity of mucus is low but Babkin (1929) and Bolton and Goodhart (1931, 1933) believed that it played a part in neutralisation of acid in the stomach. Bucher (1932) also found that gastric mucus became more viscous as the acidity is increased and less viscous in alkaline media. Although the increase in viscosity with increase in acidity can be said to be a protective mechanism, excess acid can coagulate the mucus and decrease its capacity to cover the mucosa with a continuous layer. Heatley (1959) has made the attractive suggestion that as bicarbonate containing solutions constantly exude from the
epithelial cells into the main layer a pH gradient is formed so that penetrating $\text{H}^+$ ions are neutralised and pepsin inactivated.

Virchow, in 1853, first proposed that peptic ulcer disease was due to altered blood circulation. Pavy (1863) also found it necessary to reject Bernard's theory of the protective action of the surface epithelium as the sole explanation of the resistance of the stomach to auto-digestion and a theory of protection by the alkalinity of the blood developed, often referred to as the theory of Virchow-Pavy. Bocci (1880) challenged the theory as a result of his observations that a well vascularised organ such as the spleen was digested when implanted in the stomach.

Sehrwald (1888) realised that the role of blood was not so important in neutralising acid as in preserving the life of cells. This idea that the nutritive function of the blood is required to protect from auto-digestion was also realised by Virchow (1853) who suggested that closure of the vessels in the mucosa or submucosa led to localised necrosis.

In the early days of this century another theory was suggested when Danilevski (1901) demonstrated anti-peptic activity in the gastric mucosa. However de Klug (1907) found only weak anti-peptic activity which was destroyed at low concentrations of hydrochloric acid. He reiterated Bernard's view on the protective action of mucus and the mucous secreting cells.

Around the turn of the century Fermi (1910) gave a critical appraisal of existing theories of the resistance of the stomach to auto-digestion. He rejected all the theories and proposed his own rather vague contributions - that the resistance of living cells to digestion was due to a peculiar configuration of the living protein.

In 1913 a neurogenic or spasmogenic theory of the origin of peptic ulcer was postulated by von Bergmann based on earlier work by others, notably Rokitanski (1842). According to this concept, there is a disturbance of the equilibrium of the autonomic nervous system characterised by excessive vagal stimulation. Experimental work on this subject has produced
plenty of conflicting observations but there is no doubt that experimental lesions of the brain can, but not always, result in lesions of the gastric and duodenal mucosa. The importance of these observations will be discussed later in relation to present day theories.

1.3. Modern concepts of peptic ulcer disease

1.3.1. Epidemiology

A survey of 6047 members of the general public in Great Britain (Doll et al, 1951) showed that the greatest incidence of peptic ulcer in men is in the age group 45-54 while in women the greatest incidence was not found until the age of 55. At the end of the last and the beginning of the present century peptic ulcer was prevalent in young women and this was thought to reflect itself in the incidence in the older age groups in women. A similar picture of prevalence was obtained from studies of peptic ulcer at autopsy (Watkinson, 1960). The incidence of gastric ulcer appears to increase with age but duodenal ulcer occurs more readily in the 45-64 age group than in the over 65. The figures lend weight to the theory that duodenal ulcer has increased with a probable peak in the 1950's and that the present over 65 age group had a lower incidence in their youth than the present young generation.

Men are more prone to develop peptic ulcer than women (Doll et al, 1951) and in women the incidence of peptic ulcer rises sharply after menopause (Clark, 1953) suggesting that they enjoy some measure of immunity during their reproductive years.

Peptic ulcer is found throughout the world, but there appear to be striking variations in incidence. Duodenal ulcer is more dominant in the U.S.A. than in Britain although this is apparently because of the relative scarcity of gastric ulcer in the U.S.A. (Jennison, 1938). An excess of gastric ulcer over duodenal ulcer has been recorded in only one community - a fishing village in Norway (Avery Jones, 1957). Appreciable
differences in duodenal / gastric ulcer ratio's have been recorded within Great Britain (Avery Jones, 1957) India (Dogra, 1940) and Nigeria (Aird et al, 1957).

There appears to be a relationship between gastric ulcer and social and economic background with greater preference in manual labour groups than in professional occupations (Doll et al, 1951). These social class differences may contribute to the different gastric / duodenal ulcer ratio's found in different geographical locations. Duodenal ulcer appears to be much more evenly distributed throughout the population although a higher incidence was reported among doctors and executives (Doll et al, 1951). Better diagnosis probably contributed to the higher incidence in doctors and this would suggest an underestimation in other groups.

Cleave (1962) has suggested that the recent rise in incidence of peptic ulcer is a result of greater consumption of refined food. Malhotra et al (1965) also suggested a relationship between the consumption of certain cereals and peptic ulcer although in their case the effect was attributed to the absence of roughage in the cereal rather than to the removal of protein as claimed by Cleave.

Smoking is known to diminish the healing of gastric ulcers and may also predispose to ulcer (Doll et al, 1958; Monson, 1970) but there is no sound evidence that alcohol has any effect.

The hereditary aspects of peptic ulcer lend weight to the theory that gastric ulcer and duodenal ulcer are separate diseases. Doll & Kellock (1951) found that relatives of gastric ulcer patients were more likely to develop gastric ulcer than the rest of the population while relatives of duodenal ulcer patients had a greater disposition to duodenal lesions. A greater incidence of duodenal ulcer (also Types 2 and 3 gastric) has been found in patients with blood group O phenotype (Aird et al, 1954; Clarke et al, 1955; Doll et al, 1960). A relationship between gastric ulcer and a specific blood group is less definite but Johnson's (Johnson et al, 1964) figures suggest that Type 1
gastric ulcer may be associated with blood group A.

Another genetic factor associated with an increased susceptibility to duodenal ulcer has been identified - the gene which determines whether the AB antigen is secreted in the saliva (Clarke et al, 1959). Approximately 75% of the population are 'secretors' and the incidence of ulcer disease appears to be less than in non-secretors.

Billington (1965) made the interesting observation of a rise of incidence of gastric ulcer in Australian women and a relationship between aspirin consumption and gastric ulcer in these women was later shown (Chapman & Duggan, 1969).

Although epidemiological studies have in the main yielded little there have also been encouraging findings. The observations on susceptibility to ulcer and blood group are important, not only because of the demonstration of an increased susceptibility related to genetic factors but also because they support the concept of gastric ulcer having a differing aetiological background from duodenal ulcer, prepyloric ulcer and combined gastric and duodenal ulcers. The findings of Billington (1965) are also important because they succeeded in drawing attention to an environmental causative factor of peptic ulcer.

1.3.2. Acid secretion

Acid secretion seems less important in gastric ulcer patients than in duodenal ulcer patients. Wormsley & Grossman (1965) showed that after a maximum histalog test patients with Type 1 gastric ulcer tended to have a low acid secretion while patients with Type 2 and 3 had normal or greater than normal secretion. There was great variability in the results and each group included a few patients with hypersecretion. Acid output also appears to be decreased or unchanged in patients with acute haemorrhagic gastritis or 'stress ulcers' (Ivey, 1971) or 'burn ulcers' (Harrison et al, 1973).

Rhodes (1972) has suggested that the role of acid in gastric ulcer may be to determine the site of ulceration.
Chronic gastric ulcer occurs in the antral mucosa near the junction of antral and fundic mucosa, a site which would be more exposed to excess acid than elsewhere in the mucosa (Oi et al, 1959). The predilection for the lesser curve may be determined by mechanical factors related to the musculature of the stomach wall (Oi et al, 1969).

In conclusion, although acid is necessary for an ulcer to form, the amount of acid does not appear to be of over-riding importance.

1.3.3. Pepsin

Until recently pepsin was thought not to play a major part in the aetiology of peptic ulcer. However Taylor (1959) suggested the possibility that modified pepsin, or enzymes different from pepsin, may be secreted in peptic ulcer disease and indeed found changes in peptic activity in patients with peptic ulcer suggestive of secretion of an abnormal enzyme or enzymes. Further work by Taylor (1970) revealed an increased secretion of pepsin in patients with gastric and duodenal ulcer.

Further work is of interest to assess the importance of Taylor's observation.

1.3.4. Biliary reflux

An important concept in the theory of the genesis of peptic ulcer is that reflux of the duodenal contents leads to gastritis and predisposes to gastric ulcer (Rhodes, 1972) (Fig. 1.1.). More speculatively, it has been proposed that duodenal gastric regurgitation may play a part in the genesis of duodenal ulcer (Delaney, 1972). The observations in support of duodenal reflux playing an important part in the genesis of peptic ulcer are shown in Table 1.1.

The cause of duodenal regurgitation appears to be an incompetent pylorus (Fisher & Cohen, 1972, 1973). The function of the pylorus as a sphincter is not to prevent the progress
Abnormal Motility

Gastritis

Reflux - of Bile

Fig. 1.1 Gastric ulcer in biliary reflux. Note chronic gastritis spreading upwards from the pylorus.
Table 1.1
Evidence that bile reflux may be involved in the genesis of peptic ulcer.

**GASTRIC ULCER**
1. Gastric Ulcer commonly associated with gastritis.
2. Duodenal reflux occurs more often in patients with gastric ulcer than normal.
3. Exposure of gastric mucosa to duodenal contents induces gastritis.
4. Gastritis increases the susceptibility of the mucosa to ulceration.

**DUODENAL ULCER**
1. Duodenal ulcer associated with antral gastritis.
2. Duodenal reflux occurs more often in patients with duodenal ulcer.
3. Parietal cell hyperplasia generally accompanies duodenal ulcer.
4. Exposure of antral mucosa to duodenal juice leads to antral gastritis and parietal cell hyperplasia.
of gastric contents as it is normally open, but to prevent reflux from the duodenum during duodenal cap systole for which time only it closes (Johnson, 1961). The pyloric deformity may arise as a result of duodenal ulcer (Johnson, 1957) in which case gastric ulcer usually follows or may be due to submucosal fibrosis (Rhinds, 1959) or carcinoma of the pylorus.

The evidence in favour of an involvement of bile in the genesis of gastric ulcer is strong and includes observations in man (Geall et al, 1970; Ivey et al, 1970). However all patients with gastric ulcer do not have bile in the stomach and healing of the ulcer seems to have little effect on the degree of reflux (Black et al, 1971) so that bile reflux may be an associated rather than the main aetiological factor. The evidence for a role of bile reflux in the genesis of duodenal ulcer is at the present scant.

1.3.5. Gastric motility

Dragstedt's hypothesis (Dragstedt & Woodward, 1970) of peptic ulceration is based on the concept that gastric retention leads to gastrin release with resulting hyperchlorhydria which in turn causes ulceration (Fig. 1.2.). However, although gastric stasis may in some cases be associated with peptic ulcer, there is no evidence to suggest that it is an essential accompaniment to gastric ulcer. Dragstedt's hypothesis is also weakened by the lack of hyperchlorhydria in gastric ulcer.

1.3.6. The mucosa

Many authors have suggested that decreased mucosal resistance related to gastritis may account for the ulceration. Gastritis is known to be associated with gastric ulcer (Magnus, 1952; Du Plessis, 1965; McKay & Hislop, 1966) and may be the basic disease process rather than a secondary phenomena (Gear et al, 1971). Dystrophy of the parietal and chief cells and glandular atrophy occur in gastritis (McKay & Hislop, 1966) and these changes must account, at least in part, for the acid
Fig. 1.2  Dragstedt's theory of antral stasis leading to hyperchlorhydria and gastric ulcer
hyposecretion in gastric ulcer patients.

1.3.7. Gastric mucosal barrier

The existence of a gastric mucosal barrier was first postulated by Teorell in 1933 but the concept received little attention until Davenport's (1965a) suggestion that the apparent hyposecretion of acid in patients with gastric ulcer was in fact due to back diffusion of H⁺ ions into an abnormally 'leaky' gastric mucosa.

It has been shown experimentally that the gastric mucosal barrier to H⁺ ions can be broken down by bile (Davenport et al, 1964) and fatty acids and drugs such as aspirin (Davenport, 1964). Evidence from man has suggested that increased back diffusion of hydrogen ions may contribute to apparent hyposecretion of acid in some patients with gastric ulcer (Overholt & Pollard, 1968; Chapman et al, 1968; Ivey et al, 1970), acute haemorrhagic gastritis induced by aspirin (Davenport, 1965b), acute stress ulcers (Skillman et al, 1970) and pernicious anaemia (Overholt & Pollard, 1968; Chapman et al, 1968).

1.3.8. Mucus

The structure and function of gastric mucus are described in Section 3.1.1 and the biosynthesis of its principle constituent, glycoproteins, in Section 4.1.1.

Attempts to demonstrate abnormalities of gastric mucus secretion in cases of human digestive diseases have been mostly unsuccessful so far (Piper et al, 1965; Glass et al, 1967; Robert & Henniger, 1967). Schrager (1970) however showed a decrease in mucopolysaccharides from gastric juice in patients with duodenal ulcer and correlation of the composition of mucus with the histological examination of biopsy specimens appears to be of some significance (Heinke & Berg, 1963). Domschke et al (1972) have shown that the N-acetylmuraminic acid content of gastric juice is reduced in patients with gastric ulcer. In
a recent study Donaldson et al (1974) showed that control subjects had greater sialic acid levels in gastric secretion than patients with duodenal ulcer and on the basis of estimations of volume, nitrogen, free and total sialic acid, fucose, galactose, mannose, glucose, glucosamine and galactosamine they were able to accurately group control subjects and patients with duodenal ulcer.

Some authors have suggested that sulphated polysaccharides, being peptic inhibitors, protected the gastric mucosa against proteolysis (Martin et al, 1968; Familiar, 1969). However the presence of sulphated mucosubstances in the gastric mucosa of a young and healthy patient is more than questionable (Lambert & Andre, 1972).

The greater susceptibility of non-secretors may be due in part to the structure of the components of gastric mucus. Glycoproteins isolated from the gastric juice show blood group specificity, a property intimately concerned with the terminal sugar residue (Schrager, 1970). It may be that the mucus secreted by 'non-secretors' may be less able to protect the mucosa, hence the greater incidence of peptic ulceration in this group.

In conclusion, the role of mucus in the aetiology of peptic ulcer is at present a subject for active investigation. Animal experiments have already shown that stress, bile, aspirin, corticosteroids, indomethacin and phenylbutazone all effect mucus secretion and lead to peptic ulcer (Van Geertruyden, 1961; Menguy, 1967; Lambert et al, 1971a).

1.3.9. Cell kinetics

Experimental evidence has shown that alterations in cell turnover may accompany the formation of experimental erosions. Kim et al (1967) found that an inhibition of DNA synthesis and epithelial cell production precede and accompany the development of restraint stress induced erosions in mouse gastric mucosa. Under restraint stress, a loss of RNA from the epithelial cells also occurs and Lipkin and his colleagues (Imondi et al, 1968) believe that this could be related to a decrease in the protein moiety needed for mucoprotein synthesis.
As a result of observations of excessive gastric epithelial cell loss after local application of aspirin to the human gastric mucosa Croft (1963) became interested in the exfoliation of gastric epithelial cells and developed a method of measurement based on the analysis of DNA in gastric washes (Croft et al., 1966). Using this method, abnormally high rates of cell loss were found in epithelial diseases including atrophic gastritis (Croft et al., 1966).

1.3.10. Vascular considerations

It is accepted that an adequate circulation is necessary for the integrity and function of the mucosal cells and in its absence auto-digestion occurs, perhaps explaining Hunter's "unknown living principle" (Hunter, 1772) which protects the stomach from digesting itself during life.

Many theories of ulcer genesis based on vascular alterations have been forthcoming and evidence is accumulating of vascular disturbances prior to ulceration in a number of experimental situations (documented by Pfeiffer & Sethbhakdi, 1971) and also prolonged healing of experimental ulcers due to impaired vascular generation (Umehara et al., 1971).

Clinical evidence for a vascular factor in the aetiology of peptic ulcer is very poor. More generalised vascular disease in ulcer patients than in normal subjects has been reported but generally the data are not convincing and there are contradictory findings (Pfeiffer & Sethbhakdi, 1971). However Piasecki has made the interesting observation of extramural mucosal arteries and diminished sub-mucus plexus in the most common ulcer-bearing regions of the stomach and duodenum (Piasecki, 1971, 1974). The possibility of constriction of the mucosal arteries leading to ischemia and ulceration is shown in Fig. 1.3.

1.3.11. Role of the endocrine system

The effects of the endocrine system on mucosal resistance
Fig. 1.3. Effect of muscle spasm on circulation to the mucosa. Spasm occurring at A would not occlude blood flow in the mucosal artery, a, because of collateral circulation at the level of the sub-mucosa. Spasm at B involving a mucosal artery of extramural origin might occlude the circulation in the mucosa at b. (from Piazecki, 1974)
and acid-pepsin attack are multiple and complex. Experimental
evidence provides a picture in which the result depends not only
on which hormone is acting but at what dose levels and in conjunction
with what other hormones.

Adrenal corticosteroids are often implicated in the
disease process but at present there is no direct evidence
linking peptic ulcer with adrenal function (Avery Jones et al,
1968).

Sex hormones appear to play a part in the development
of peptic ulcer as peptic ulcer occurs dominantly in males and
the relative immunity enjoyed by women almost disappears at
the onset of menopause (Clark, 1953).

Peptic ulcer can arise from polyendocrine tumours,
the glands most often involved being the pancreas, the parathyroids,
pituitary and adrenals. The mechanism of ulcer formation is not
understood except in the case of the Zollinger-Ellison syndrome
(Zollinger & Ellison, 1955) caused by a pancreatic tumour which
secretes enormous amounts of gastrin (Gregory et al, 1960).

Although there is little evidence to implicate the
gastrointestinal hormones in peptic ulcer, recent research has
indicated that they are involved not only in control of motility
and secretions of the gastrointestinal tract but also in regulation
of DNA, RNA and protein synthesis. As our knowledge of gastrin,
secretin, cholecystokinin and other, as yet putative, gastrointestinal
hormones increases with the advent of radioimmunoassay it is
possible that a major role in peptic ulcer may be discovered.
Although Dragstedt's theory of antral stasis and hypergastrinaemia
is not supported because the elevated serum gastrin levels seen
in some patients with gastric ulcer are associated with decreased
acid secretion, it has been suggested that an abnormal gastrin
response may be a factor in duodenal ulceration (Berson & Yalow, 1971).

1.3.13. Role of the central nervous system

Bykov and Kurzin (1954) developed the corticovisceral
theory of peptic ulcer formation which explains the genesis of
Peptic ulcer on the basis of a pathological integration of the relationship between cortical perception, the environment and the internal organs. Balo (1963) submitted evidence that discernable lesions were demonstrated in the central or peripheral central nervous system in the majority of peptic ulcer cases studied.

Acute haemorrhagic gastric lesions, often referred to as "stress ulcers" are often seen in severely ill patients after major surgery or trauma (Flowers et al, 1970). These are sometimes confused with classical Cushing's (Cushing, 1932) ulcers (neurosurgical) or Curling's (Curling, 1842) ulcers (burns) in which there is a deeper lesion resembling a normal peptic ulcer.

Psychological factors seem to play an important part in the development of peptic ulcer but the mechanisms involved are not clear. In general, it has been stated that chronic anxiety and stress or multiple episodes of acute anxiety and stress can produce a gastric hypersecretory state which may predispose to ulceration (Richman, 1971). The role of the parasympathetic nervous system and the adrenal gland appear to be particularly important. However many experimenters have produced conflicting data and there is need for further research to clarify the basic mechanisms. More importance should also be attached to mucosal and vascular factors.

1.3.13. Drugs and peptic ulcer

It is well known that, clinically, many drugs exhibit gastrointestinal irritation as one of their side effects. The use of otherwise excellent anti-inflammatory agents such as aspirin, phenylbutazone, indomethacin and corticosteroids is often restricted due to the chances of producing acute gastric and intestinal lesions and even perforation. Interesting observations have recently been made by Macdonald (1973) who found chronic ulcers associated with aspirin ingestion in an otherwise normal stomach. Chronic gastric ulcers are usually
associated with gastritis and Macdonald (1973) concluded 'the results add further evidence that these (aspirin containing) drugs are ulcerogenic'.

1.3.14. Treatment of peptic ulcer

Conventional medical treatment of peptic ulcer has in the past relied mainly on procedures aimed at neutralising acid in the stomach. Doll (1964) carried out a carefully controlled trial of measures to which had been attributed activity in healing peptic ulcers. The treatments which Doll found to be ineffective included bland diet, low fat diet, milk drip, milk drip plus alkali, phenobarbitone, belladona, ascorbic acid, cabbage juice, Robaden and a diet of added brains. Antacids have been a popular measure in gastric ulcer therapy but clinical trials have shown that they have no value in the healing of gastric ulcer (Baume & Hunt, 1969). They are still used however for the relief of pain which they produce in spite of many toxic reactions which may occur (Piper & Heap, 1972).

Anticholinergic drugs have been prescribed in peptic ulcer therapy with the aim of producing a medical vagotomy but the results from clinical trials are not convincing. A wide spectrum of side effects such as dry mouth, blurred vision, difficulty in micturition and impotence occur with this treatment.

Various other approaches to medical therapy such as Depepsen (pepsin inhibitor) and cholestyramine (sequesters bile acids) have been tried but the evidence from clinical trials is not promising (Rhodes, 1972). Claims made for a deglycyrrhizinized liquorice preparation (Caved - S) were not substantiated in a well-controlled trial (Engqvist et al, 1973). Recent evidence has suggested that prostaglandins (Fung et al, 1974b) and histamine H₂ - receptor antagonists (Milton-Thompson et al, 1974) may have some effect but careful, well controlled trials are necessary before any conclusions can be reached as to their value in peptic ulcer therapy.

Doll and his colleagues have shown that measures

* a proprietary extract of gastrointestinal tissue
which do successfully promote ulcer healing are bed rest (Doll & Pygott, 1952) cessation of smoking (Doll et al, 1958) and carbenoxolone sodium (Doll et al, 1962). The reasons for the beneficial effects of bed rest are not clear; the traditional interpretation of this has been relief of stress but it has also been suggested that bile reflux is less in the supine patient (Capper, 1967; Flint & Grech, 1970).

The introduction of carbenoxolone sodium therapy into the treatment of gastric ulcer has probably been the biggest single advance in peptic ulcer therapy. The original observations made by Doll et al (1962) have been confirmed in many clinical trials since (Sircus, 1972; Avery Jones & Parke, 1975). Early trials with carbenoxolone failed to demonstrate its efficacy in patients with duodenal ulcer. This was thought to be due to the fact that carbenoxolone exerts its effects mainly by direct topical action and absorption by the stomach prevented it reaching the duodenal mucosa in sufficient amounts. A gelatine capsule was therefore devised to deliver carbenoxolone directly into the duodenum, thus by-passing the stomach. The results with this preparation of carbenoxolone for the treatment of duodenal ulcer have shown that it induces earlier healing of duodenal ulcer (Hunt, 1972; Amure, 1970; Marcos Perez, 1970). Side effects such as oedema, hypertension, hypokaleamia, and, very rarely, myoglobinuria, and myasthenia, sometimes occur with carbenoxolone therapy but these are easily controlled if proper care and attention is given (Sircus, 1972).

Consideration will be given to the mode of action of carbenoxolone in a later Chapter (Section 6.1.1.).

The usual indications for surgery are 1) failed medical treatment 2) pyloric stenosis and hour glass stomach and 3) haemorrhage. Partial gastrectomy is the operation of choice for gastric ulcer and for duodenal ulcer vagotomy with a drainage procedure is preferred.
CHAPTER 2

"GROSS AND HISTOPATHOLOGICAL STUDIES OF GASTRIC ULCERS AND PROSIONS"

2.1. INTRODUCTION

2.1.1. Stress ulcers

2.1.2. Methods for production of experimental chronic ulcers

2.1.3. Drug-induced experimental ulcer

2.2. EXPERIMENTAL

2.3. RESULTS

2.4. CONCLUSIONS
2.1. INTRODUCTION

The main purposes of experimental ulcers are to study mechanisms of pathogenesis and healing in peptic ulcer and to evaluate potential therapeutic agents. Many ulcer models have been developed for gastric and duodenal ulcers including surgical methods (Mann-Williamson preparation, Mann & Williamson, 1923) pylorus ligation (Shay et al, 1945), various means of trauma and stress, physical or chemical trauma and a variety of drugs including corticosteroids. Knowledge of the ways in which erosions or ulceration might develop has been obtained using these methods and it is likely that studies involving detailed microscopic and biochemical analysis of the mucosa in normal and in ulcerated animals will provide further basic data necessary for understanding of the disease.

The models used in the present work will be briefly reviewed.

2.1.1. Stress ulcers

The most popular method used to stress animals for the production of gastric lesions has been immobilization or restraint. The first report of the use of restraint as a stress was in 1936 when Selye immobilized rats by spinal cord transection and observed clearly-defined gastric ulcers. Later, Rossi et al (1956) were the first to use restraint as a method to produce experimental gastric ulcer rather than a general 'stressor'. Reports from Bonfils and his group in 1959 (Bonfils et al, 1959) and from Brodie and Hanson in 1960 were the first of many from these two groups of workers demonstrating the ease, reliability and drug sensitivity of this preparation.

The lesions produced by restraint stress penetrate the muscularis mucosa and thus are more properly termed 'erosions' than ulcers. The pathogenesis of stress ulcers or erosions is not clearly understood but evidence suggests that more than one factor is involved. Findings which may have relevance to the pathogenesis with examples of references are summarised in Table 2.1.
<table>
<thead>
<tr>
<th>Findings</th>
<th>Reference (Example)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Vascular changes</td>
<td>Guth (1972)</td>
</tr>
<tr>
<td>2. Acid hypersecretion</td>
<td>Guth &amp; Kozbur (1968)</td>
</tr>
<tr>
<td>3. Acid hypersecretion not involved</td>
<td>Brodie &amp; Hocke (1971)</td>
</tr>
<tr>
<td>4. Impaired mucus synthesis</td>
<td>Ludwig &amp; Lipkin (1969)</td>
</tr>
<tr>
<td>5. Vagal over-activity</td>
<td>Goldman &amp; Rosoff (1968)</td>
</tr>
<tr>
<td>7. No back diffusion of $H^+$ions</td>
<td>Gerety &amp; Guth (1972)</td>
</tr>
<tr>
<td>8. Impaired cell regeneration</td>
<td>Kim et al (1967)</td>
</tr>
<tr>
<td>10. Mast cell degranulation</td>
<td>Rasanen (1963)</td>
</tr>
<tr>
<td>11. No direct role for mast cell degranulation</td>
<td>Guth (1971)</td>
</tr>
</tbody>
</table>

Table 2.1

Findings relevant to the pathogenesis of stress ulcer.
As an experimental ulcer model the main advantages of this preparation are that no surgical intervention is required and that the lesions are produced by a physiologically-produced stress rather than ulcers or erosions produced by chemical or mechanical means. However, the similarity between the experimental lesions and clinical gastric ulcer must not be overestimated and the nature of the lesions makes them more akin to those occurring in acute gastric mucosal bleeding and haemorrhagic gastritis in humans than in chronic gastric ulcer.

2.1.2. Methods for production of experimental chronic ulcers

One of the main disadvantages of most experimental ulcer models is that the experimental lesions are fairly superficial and heal without scarring. There have been relatively few studies which attempt to produce a situation similar to that found in man, although the chronicity of human peptic ulcer is one of the major problems of the disease.

Skoryna et al (1958) produced penetrating gastric ulcers by thermocautery of the gastric mucosa and further reports from the same laboratory show that cortisone administered to the rat resulted in the early penetration and enlargement of these ulcers (Khan et al, 1961a) and in the re-ulceration and penetration of healed ones (Khan et al, 1961b).

In 1963, Umehara and colleagues (1963) reported a new method for the production of chronic gastric ulcer. The procedure consisted of clamping the stomach with a metal plate for 24 hours and administering cortisone for 7 days from the first operation day. Ulcers so produced took up to 13 weeks to heal and studies on the pathogenesis and suitability of the model for drug screening have been carried out (Umehara et al, 1971).

Takagi et al (1969) developed a method for inducing chronic gastric ulcer in rats by means of submucosal injections of acetic acid and Okabe et al (1970) modified the method for induction of gastric and duodenal ulcers. The ulcers presented diminish in size rapidly during the early phase of recovery, but persist for up to 200 days, probably by repeated healing and
re-aggravation (Okabe & Pfeiffer, 1971).

The methods described above all have the advantage of resembling human chronic peptic ulcers in that the histological appearance of the ulcers is similar to that in humans, the ulcers appear to be truly chronic, with episodes of healing and re-exacerbation as occurs in the clinical disease, and they lend themselves to the possibility of investigating the effects of drugs on healing rather than the production of ulcers.

The disadvantages are that the methods of production are completely unphysiological and studies of the factors leading to ulceration are impossible.

### 2.1.3. Drug-induced experimental ulcer

The object of drug-induced ulceration as an experimental model is twofold: first to predict the ulcerogenic properties of new potential therapeutic agents and, second to test the effect of anti-ulcer agents against these drug-induced ulcers.

The studies carried out on ulcerogenic drugs are numerous and many explanations have been put forward as to the mechanisms of action of individual compounds. Table 2.2 shows the drugs most commonly used to produce experimental ulcers and lists the probable mechanisms of their action.
Table 2.2.
Possible mechanisms of action of commonly used drug-induced experimental ulcer models.

A. Corticosteroids
1. Increased acid-pepsin secretion (Vanov & Mildsevic, 1962)
2. Breakdown of mucosal barrier (Robert & Nezamis, 1963)
3. Decrease of mucosal resistance (Vanov & Mildsevic, 1962)
4. Interference with healing and vascular and metabolic disturbance. (Segal, 1960)
5. Decrease of mucin secretion (Robert & Nezamis, 1963)
6. Impairment of arterial supply to mucosa (Weinshelbaum & Ferguson, 1963)
7. Reduced rate of regeneration of epithelial cells (Max & Menguy, 1970)

B. Salicylates
1. Increase in shedding of surface epithelial cells (Max & Menguy, 1969, 1970)
2. Back diffusion of hydrogen ions (Davenport, 1967)
3. Lowered gastric mucosal blood flow (Takagi & Kawashima, 1969)
4. Decrease of mucus (Menguy & Masters, 1965)
5. Bile in stomach necessary (Djahanguiri et al, 1973)
6. Sympathetic nervous system involved (Hemmati et al, 1973)

C. Phenylbutazone
1. Decrease in mucus production (Menguy & Desbaillets, 1967)
2. Increased shedding of surface epithelial cells (Max & Menguy, 1969, 1970)
3. Not caused by hyperacidity (Menguy & Desbaillets, 1967)
4. Lysosomal damage (Lewis et al, 1971)
5. Vascular changes (Barroy et al, 1970)
2.2. EXPERIMENTAL

2.2.1. Materials

The rats used in these studies were from our own random-bred colony of Wistar rats (Biorex Wistar) originally derived from S.P.F. Wistar rats supplied by A.Tuck & Son Ltd., Essex. The rats were housed in wire mesh cages in animal house conditions of 22°C and 60% relative humidity with food (Dixon's FFG (M) diet) and water allowed ad lib unless the experimental protocol required alteration of these conditions.

Other animals used, male and female albino guinea pigs, approx. bodyweight 500 g or over, and male and female cats 1.5 - 3.5 kg bodyweight were kept under similar conditions.

The electrocautery apparatus was specifically constructed for these studies by Mr. Twinn, Department of Physics, Guy's Hospital (Fig. 2.1.). The cautery disc, 4 mm diameter was fitted to the end of a portex nylon intravenous cannula (2.1 mm external diameter).

For anaesthesia, either anaesthetic ether (May & Baker) or phenobarbitone sodium (Nembutal, Abbott Laboratories) were used.

The following were used in histological processing: 2 or 10% formol saline (2 or 10% formalin in 0.9% NaCl); acid alcohol (1% HCl in 70% alcohol); 1% eosin (George T. Gurr Ltd.); D.P.X. mounting medium (Raymond A. Lamb). Harris's Alum Haematoxylin was prepared by dissolving 2.5 g haematoxylin (B.D.H.) in 50 ml of absolute alcohol and mixing with 50 g of potassium alum dissolved in distilled water. The mixture was heated to boiling point and 1.5 g mercuric acid added. The solution was cooled rapidly by plunging the flask into cold water.

The following drugs were dissolved or suspended in 0.1% aqueous Tween 80, 0.1% Tween 80 in 0.9% NaCl or isopropyl myristate: prednisolone, cortisone acetate, phenylbutazone and aspirin (all kindly supplied by Biorex Laboratories Ltd.).

Unless otherwise indicated all chemicals and solvents were obtained from B.D.H. or other usual laboratory suppliers.
Circuit diagram for diathermy apparatus
2.2.2. Preparation and staining of samples for histological evaluation

After adequate fixation in formol saline, tissues were dehydrated in alcohol, cleared in chloroform and impregnated with wax by automated Histokinette, embedded in paraffin wax blocks and sections 5 μ thick cut on an M.S.E. sledge microtome. The sections were attached to slides by floating them on warm water. Wax was removed from the sections by placing them in xylene and hydration was carried out prior to staining. The slides were then transferred to Harris's Alum Haematoxylin where they were left for 15 minutes. After draining off excess haematoxylin, the slides were washed until the sections became blue (when first removed from the haematoxylin they are pink). This change of colour has caused this stage to be called 'bluing'. Sections were then dipped in acid alcohol for a few seconds and returned to water until they were blue again. Sections which have been differentiated and 'blued' were transferred to 1% eosin for 2-4 minutes to counterstain them and then washed. After draining, the sections were dehydrated in alcohol, cleared in xylene and mounted under a coverslip using D.P.X. mounting medium.

2.2.3. Restraint stress

The method of restraint was based on that of Brodie & Hanson (1960) and is described below.

The rats were lightly anaesthetised with ether and placed in wire mesh envelopes which were moulded around the animals and held in place with wire staples so that the rats were unable to move. The rats were allowed to recover from anaesthesia and then placed in separate plastic cages in a temperature controlled room.

At the end of the restraint period, the rats were removed from their meshes and sacrificed by killing with ether. The stomachs were removed and filled with 2% formol saline as a fixative. After fixation, the stomachs were cut along the
greater curvature, pinned to a dissection board and examined under a stereoscopic microscope (x 8.75). The severity of the erosions was expressed on an arbitrary scale of 0 to 6 as follows: 0 = no damage; 1 = blood in lumen; 2 = pinpoint erosions; 3 = 1-5 small erosions (<2 mm); 4 = >5 small erosions; 5 = 1-3 large erosions (>2 mm); 6 = >3 large erosions.

For histology studies, 5μ sections were taken and stained with haematoxylin and eosin (H & E), until a sufficient number of lesions that were identified under the stereoscopic microscope were confirmed by histological examination.

The effect of a single dose of prednisolone on the susceptibility to stress erosions was investigated using a 24 hour restraint period at 15 to 18°C without prior fasting. Prednisolone was administered intramuscularly in saline at a dose of 75 mg/kg either 18 hours before the commencement of restraint or immediately after the restraint period and the rats were sacrificed at various periods after restraint.

The effect of repeated restraint plus cortisone administration was investigated by restraining rats for 18 hours each day for 4 days. During the 6 hours between one restraint period and the next, the rats were given food and water ad lib. Just before each restraint period the rats were injected with 75 mg/kg intramuscularly of cortisone acetate in isopropyl myristate. The rats were sacrificed on the fifth day, i.e. after 4 periods of restraint and the stomachs excised, fixed and examined as described above. 5μ sections of stomach were cut and stained with haematoxylin & eosin for histological examination.

2.2.4. Physical trauma

(a) Electrocautery

Female Biorex Wistar rats (bodyweight 200 g or over) or, in one experiment male and female albino guinea pigs (approx. 500 g bodyweight) were used in these experiments. The animals were either deprived of food the night prior to the operation
or used in the afternoon, when the stomach is in any case relatively empty.

The procedure was as follows: the stomach was exposed through a laparotomy incision made under ether anaesthesia. Ulcers were produced by a specially devised cautery (Section 2.2.1.). The cautery disc was introduced via the oesophagus into the stomach and applied to the body mucosa to produce an ulcer.

After sacrifice of the animals, the stomachs were excised and distended with formal saline and later examined under a stereomicroscope. As the ulcers obtained were sometimes oval shaped, rather than circular, the ulcer index was taken as the product of the length and width of the ulcer. Histological sections through the ulcers were cut at 5μ and stained with haematoxylin & eosin.

Cortisone acetate, 75 mg/kg intramuscular, was administered to rats for 4 days beginning on the day of ulcer production.

Phenylbutazone was given orally at a dose of 100 mg/kg for three days, beginning on the day of ulcer production.

(b) Acetic acid ulcers

Male BX Wistar rats, bodyweight 200-250 g, were anaesthetised with ether, the stomach exposed by laparotomy, and 0.05 ml of 30% acetic acid injected subserosally into the stomach wall.

The injection was taken as successful if a blister with little or no leakage of acetic acid was obtained. The animals were discarded if the injection failed to produce the characteristic blister. The animals were not starved prior to the operation but the ulcer preparations were made in the afternoon when the stomach is relatively empty.

After sacrifice, the stomach was removed and filled with approximately 10 ml of 2% formal saline as fixative.

As the ulcers produced by this method are sometimes oval, rather than circular, the product of the length and width was taken as the ulcer index.
The same method was used for guinea pigs except that Nembutal (35 mg/kg i.p.) was used as anaesthetic.

2.2.5. Anti-inflammatory drugs

a) Phenylbutazone

Female Wistar rats (150-200 g) were deprived of food on the first day and on the following day were given two oral doses (100 mg/kg each) of phenylbutazone (10 am and 6 pm). The rats were sacrificed the following morning and the stomachs distended and fixed with formol saline and histological studies carried out on 5 μ sections stained with haematoxylin & eosin.

b) Aspirin

Erosions were induced in female (100-200 g) rats by a single oral administration of aspirin (200 mg/kg) and the effect of food deprivation 18 hours prior to aspirin administration investigated. Erosions were assessed as described in Section 2.2.3.

Cats were prepared according to the method of Alphin & Droppleman (1971) in which the stomach is opened and placed in a chamber perfused with artificial gastric juice. During the perfusion with gastric juice, test substances were applied directly on the mucosa with a tuberculin syringe (0.1 ml) and confined to relatively small areas by means of perspex rings (2.5 cm diameter).

In a series of experiments using 7 cats, aspirin, 18 mg (in 20% gum acacia) was applied to areas of the mucosa and the mucosa observed for the development of bleeding points and/or erosions.

2.2.6. Corticosteroids

The method is based on that of Robert & Nezamis (1958). The rats were caged individually and fasted for the five days
of the experiment, being allowed water *ad lib.* During the fasting period prednisolone, suspended in isopropyl myristate (40 mg/ml), was injected alternately into the right and left thigh at a dose of 40 mg/kg daily for 4 days. On the fifth day the animals were sacrificed and the stomachs removed and distended in 2% formol saline as a fixative. The stomachs were examined by means of direct illumination and the erosion index was taken as the total area of erosion. Sections were embedded in paraffin, cut at 5µ and stained with haematoxylin & eosin for histological examination.
2.3. RESULTS

2.3.1. Stress ulcers

(a) Pathology and histopathology

The lesions produced by restraint stress appeared on gross examination to vary from areas of diffuse erythema to round minute pin point erosions and to linear erosions sometimes over 4 mm long (Figs. 2.2 & 2.3). The edges of the erosions were sharply demarcated and their bases usually haemorrhagic. They were found exclusively in the glandular portion of the rat stomach, almost always in the corpus. Only very occasionally did an antral lesion present itself. Histologically the eroded mucosa was necrotic, being eosinophilic and containing only nuclear fragments (Figs. 2.4 & 2.5). The erosions were accompanied and preceded by areas of oedema and dilatation and congestion of capillaries. Interstitial haemorrhage also occurred from rupture of the congested vessels. Occasionally thrombi associated with superficial cell necrosis were observed. A neutrophilic reaction was observed, mainly in the later stages of erosion formation. Another common finding was enlargement of the lumen of the gastric pits, with flattened epithelial cells present.

Most of the erosions were superficial with about half of the mucosa appearing necrotic but on occasions erosions extending to the muscularis mucosa with neutrophilic invasion were observed. The lesions were never found to penetrate into the muscularis mucosa.

Changes in the antrum were less marked. Occasionally superficial erosions with dilation of the blood vessels was observed. Widening of the lumen of the antral glands was also associated with stress.

(b) Factors found to affect the severity of stress erosions

Reproducible results were only obtained when care
Fig. 2.2

Haemorrhagic erosions in rat stomach induced by restraint stress

Fig. 2.3
Fig. 2.4

Large stress erosions in body mucosa of rat with slight inflammatory cell infiltration (H & E, x 150).

Fig. 2.5

Thrombosis of mucosal vessels with superficial cell necrosis after restraint (H & E, x 150).
was taken to control all the variables possible. The effect of various factors on the incidence and severity of erosions is shown in Table 2.3.

(c) Recovery from stress erosions

The lesions healed quickly and the stomachs were almost back to normal after 7 days. Examination of the stomachs 14 days after production of the stress erosions showed that they healed without any visible scarring.

Histological examination of the stomachs 7 days after stress showed that re-epithelialisation was almost complete. The lesion was composed of connective tissue with round cell infiltration. No chief or peptic cells were present in the regenerated epithelium.

14 days after stress, the defect was completely repaired by regenerated epithelium. The mucosa of this region differed from the normal mucosa in that no chief or parietal cells were present and the appearance was similar to antral mucosa.

(d) Stress plus prednisolone

A single subcutaneous administration of prednisolone 75 mg/kg immediately after the restraint period did not significantly alter the healing times of the erosions. The same dose of prednisolone administered 18 hours before the restraint period appears to have actually delayed the appearance of stress erosions.

(e) Repeated stress plus cortisone

After receiving 18 hour periods of restraint plus cortisone acetate 75 mg/kg daily for 4 days gross examination of the animals revealed varying degrees of pathology in the glandular stomach from diffuse erythema to deep erosion or ulceration of the stomach. No gross changes were observed in the rumen.

In most of the rats, lesions similar in appearance
Table 2.3.
Factors found to affect the severity of stress erosions

<table>
<thead>
<tr>
<th>Factor</th>
<th>Remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td>Temperature</td>
<td>Lower temperatures resulted in a greater incidence and severity of ulceration. At very low temperature (2-5°C) high mortality and low incidence of ulceration obtained.</td>
</tr>
<tr>
<td>Period of restraint</td>
<td>Incidence of erosions reached a maximum sooner than erosion index. By various combinations of restraint and temperature can choose a method with an incidence and severity as required.</td>
</tr>
<tr>
<td>Sex and bodyweight</td>
<td>Females were more susceptible than males and immature animals (50g) less susceptible than mature ones (150g and over). In male rats the severity of erosions reached a maximum at around 150g bodyweight while in female rats the severity kept increasing to the greatest weight used, 250g.</td>
</tr>
<tr>
<td>Season</td>
<td>Greater incidence and severity of erosions in the Winter as opposed to Spring and Summer months.</td>
</tr>
<tr>
<td>Feeding</td>
<td>Fasted rats more susceptible than non-fasted.</td>
</tr>
</tbody>
</table>
to those obtained from a single exposure to stress were observed. Most of the lesions were haemorrhagic but there were no traces of blood in the others, indicating that they had appeared at an earlier time. No erosions were seen in the antral region in the glandular stomach of any of the rats.

In five out of the 15 rats deeper erosions than had been encountered previously were obtained. These lesions were linear, up to 5 mm long and 2 mm wide and were all located in the glandular stomach close to the transverse ridge.

Histology of these deeper lesions revealed severe erosion of the glandular epithelium. In some cases the mucosa was completely destroyed but in no cases was penetration through the muscularis mucosa observed.

2.3.2. **Electrocautery ulcers**

(a) **Pathology and histopathology**

The electrocautery produced full thickness areas of necrosis which, after about 48 hours, resulted in deep ulcers penetrating through the full thickness of the gastric wall (Figs. 2.6 & 2.7). The ulcers were frequently found to be supported by adherence to neighbouring structures, mostly pancreas and, less commonly, liver. In some cases perforation was often confined by the liver, allowing the animal to survive.

In the first 2-3 weeks after the production of the ulcer the healing was rapid and the ulcers decreased in size from their original 7 mm diameter (Fig. 2.8). However the ulcers failed to heal completely in 4 weeks. On examination of 4 stomachs 70 days after ulceration, one exhibited a completely healed ulcer, one almost healed, while the other two presented largish ulcers.

Detailed histological study of the formation and healing of the ulcers revealed that within 2 days of the cautery the damage penetrated through the entire stomach wall with the muscularis mucosa and outer muscle coats completely destroyed.
Fig. 2.6

Electrocautery ulcer in rat stomach 5 days after production

Fig. 2.7

Detail of electrocautery in Fig. 2.6
Effect of cortisone on healing of cautery-induced ulcers in rats

Rats were sacrificed at various intervals after induction of electrocautery ulcers. Each point is the mean of at least 4 rats.
A massive inflammatory cell infiltration into the necrotic tissue was observed. Healing by proliferation of granulation tissue and new capillaries began around 6 days after the electocautery and by 10 days thick fibrous floors had formed, often overlain with a band of necrotic tissue. Beneath the necrotic layer was healthy granulation tissue, with numerous fibroblasts, inflammatory cells and new capillaries. Epithelial regeneration was also observed at this stage although in some cases this amounted to only a single layer, one cell deep, migrating across the floor. In others the regenerating epithelium was more complex. From 30 days after cauterity onwards the general histological appearance of the ulcers was one of mature fibrous tissue and a round cell infiltration in the floor with varying degrees of epithelial regeneration (Fig. 2.9). Thick bundles of collagen running vertically were observed in some ulcers 55 and 70 days after cauterity.

(b) Effects of cortisone

As can be seen from Fig. 2.8 intramuscular cortisone acetate 75 mg/kg daily for 4 days beginning on the day of ulcer production resulted in greater penetration and enlargement of the ulcers in initial stages. The ulcers remained larger throughout the period observed. Histological examination of the ulcers after cauterity showed no qualitative differences between cortisone-treated animals and those receiving cauterity alone, apart from trapped mucosal glands. However, the unhealed ulcers in the cortisone-treated animals tended to be larger and thicker and the existence of three layers in the ulcer floor was often apparent: 1) a thin layer of necrotic tissue; 2) healthy granulation tissue with numerous fibroblasts, chronic inflammatory cells and new capillaries. Bundles of collagen running vertically were sometimes observed; 3) a layer of mature fibrous tissue.

In addition, a complex pattern of epithelial regeneration with mucosal glands trapped deep down in the ulcer floor was sometimes observed in the cortisone-treated animals.
Extensive regenerating epithelium 30 days after production of an electrocautery ulcer (H & E, x 60).
(c) **Effect of phenylbutazone**

Phenylbutazone, 100 mg/kg orally per day, administered on days 0-2 had no significant effect on the healing of cautery ulcers as measured on days 7 and 15 although resulting in characteristic erosions of the rat glandular stomach (Table 2.4).

(d) **Electrocautery ulcers in guinea pigs**

An experiment to determine the healing characteristics of cautery ulcers in guinea pigs showed that complete healing took about only 2 weeks.

2.3.3. **Acetic acid ulcers**

In appearance and in the early days of healing these ulcers resemble those induced by electrocautery (Figs. 2.10 & 2.11). With guinea pigs the healing appears to be much slower than in rats in the first 10 days but is rapid between 10 and 20 days after the operation to the extent that the ulcers presented on Day 20 were almost completely healed. This is similar to the situation obtained with cautery ulcers, where healing is much faster in guinea pigs than in rats.

2.3.4. **Anti-inflammatory drugs**

(a) **Phenylbutazone** The group receiving two oral doses of phenylbutazone 100 mg/kg showed 100% incidence of erosions. The lesions produced were superficial appearing as erosions of the glandular mucosa associated with areas of oedema without penetration into the muscularis mucosa (Fig. 2.12). In this respect they were similar to those produced by stress.

(b) **Aspirin** The single oral administration of aspirin (200 mg/kg) produced 100% incidence of erosions when administered to rats previously fasted for 18 hours. The incidence and
Table 2.4.

Effect of phenylbutazone on the healing of cautery ulcer.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Day of sacrifice</th>
<th>No. of rats</th>
<th>Ulceration Mean ulcer index mm² ± s.e.m.</th>
<th>PBZ erosions Mean erosion index mm² ± s.e.m.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cautery alone</td>
<td>7</td>
<td>4</td>
<td>29 ± 4</td>
<td>0</td>
</tr>
<tr>
<td>Cautery + phenylbutazone</td>
<td>7</td>
<td>4</td>
<td>24 ± 2</td>
<td>11 ± 3</td>
</tr>
<tr>
<td>Cautery alone</td>
<td>15</td>
<td>5</td>
<td>9 ± 3</td>
<td>0</td>
</tr>
<tr>
<td>Cautery + phenylbutazone</td>
<td>15</td>
<td>3</td>
<td>6 ± 2</td>
<td>8 ± 3</td>
</tr>
</tbody>
</table>

Phenylbutazone was administered at a dose of 100mg/kg orally to the rats for three days beginning on the day of ulcer production. There are no significant differences in the electrocautery ulcers between phenylbutazone-treated rats and those which received electrocautery alone.
Fig. 2.10

Acetic acid ulcer 15 days after production

Fig. 2.11

Histological section through acetic acid ulcer showing complex regenerated epithelium over a floor of granulation and connective tissue (H & E, x 90).
Acute haemorrhagic gastric mucosal erosion after oral phenylbutazone in the rat.
severity of erosions was significantly less in non-fasted rats.

The lesions induced by aspirin were typical acute haemorrhagic erosions varying from pin points to large erosions over 4 mm long (Fig. 2.13). The lesions failed to penetrate through the muscularis mucosa.

In the cat chamber experiments, erosions began to appear after about 10-15 minutes and increased in number in a linear fashion up until about 90 minutes after aspirin administration. The lesions developed from minute bleeding points to areas of gross haemorrhagic erosions (Fig. 2.14).

2.3.5. Corticosteroids

In animals receiving 8 mg of prednisolone intramuscularly per day for 4 days 100% incidence of rats with erosions was observed (Fig. 2.15). Without food deprivation during the period of prednisolone administration, the effect of prednisolone was greatly reduced, with pin point erosions only being observed. Food-deprivation alone also resulted in pin point erosions in some of the rats.

Histologically, erosion and thinning of the gastric mucosa was seen. Widespread oedema and vascular changes ranging from dilation and congestion of blood vessels to severe haemorrhage was observed. The lumen of the pits was widened. The erosions varied in severity from superficial necrosis of half the mucosa to extension of the lesion to the muscularis mucosa. Necrotic debris and round cell infiltration were associated with deep erosions.

In one severe erosion it appeared that epithelial regeneration was starting, with a single layer of cells beginning to migrate across the necrotic base (Fig. 2.16).

Observation of the recovery period confirmed the acute nature of the lesions. After 30 days the stomachs were almost back to normal and the epithelial covering was replaced.

Histologically, the lesion was covered by regenerated
Aspirin-induced acute haemorrhagic erosions in rat stomach

Haemorrhagic erosion in cat stomach after topical administration of aspirin.
Haemorrhagic gastric mucosal erosion induced by prednisolone

Prednisolone erosion. Epithelial regeneration from the margins of the erosion can be seen. (H & E, x 150).
epithelium 10 days after. However some congestion of mucosal blood vessels was still apparent and the regenerated epithelium was less complex appearing as simple down-growths with no gastric glands. Parietal and peptic cells were not present. 30 days after, the mucosa appeared completely normal apart from slight capillary dilatation and congestion.
2.4. **CONCLUSIONS**

2.4.1. **Restraint stress**

The gastric damage produced by restraint stress was found to be similar to that reported by Brodie (1962) and Bonfils et al (1959). The pathological and histopathological examinations of the stomachs of rats after restraint stress gave indications of some of the changes which were occurring as the erosions developed, i.e. oedema, capillary congestion, cell necrosis etc. but it was not possible to establish the factors directly responsible for the initiation of the lesions. These factors are probably complex as can be seen from Table 2.1 which lists findings which may have relevance to the pathogenesis of stress ulcers. Further studies on cellular, biochemical and molecular mechanisms are indicated.

The restraint method produces a rapid, simple technique for the production of gastric erosions in rats and one which lends itself to the study of mechanisms of lesion formation and of factors which influence their formation. However, the reproducibility of erosion formation in rats depends on careful control of experimental conditions as the present work shows.

Healing of the stress erosions appears to take place by migration and regeneration of the epithelial cells from adjacent or undamaged tissue. The regenerated epithelium can be distinguished from normal epithelium as there are no parietal or zymogen cells present and the glands are less complex. Regeneration of parietal and zymogen cells in the rat has been reported to take three to five weeks (Myhre, 1956; Hunt, 1958). The rapid spontaneous healing of stress erosions is one of the disadvantages of this method as it is difficult to determine the effects of procedures which accelerate ulcer healing.

Attempts to produce a lesion taking a longer time to heal by the administration of a corticosteroid either before or after the restraint period were not successful and, in fact, administration of prednisolone before restraint appears to have delayed the appearance of erosions. From this it appears that
prolonged administration of corticosteroids would be necessary to significantly alter the healing rate of stress erosions.

The literature confirms that there is no evidence that the administration of corticosteroids either before or after restraint will alter the nature of the lesion although prolonged administration may delay their healing.

The attempts to produce a chronic-type lesion by repeated restraint along with corticosteroid administration resulted in more severe erosions but the lesion did not have the characteristics of chronic ulcers — the muscularis mucosa remained intact and healing took place by epithelial regeneration. Brodie & Hanson (1960) also found that repeated periods of 18 hours restraint increased the severity of erosions although not producing a chronic-type ulcer while repeated periods of 4-6 hours restraint were found to actually reduce the incidence and severity of erosions (Guth & Mendick, 1964; Martin et al, 1970).

2.4.2. Electrocautery ulcers

The electrocautery method in the rat produces full-thickness experimental ulcers, resembling human chronic gastric ulcer in their histological appearance. This method is similar to that of Skoryna et al (1958) but these authors found that the ulcers healed in about 28 days whereas in the present study ulcers were still present 90 days after induction. The healing of the ulcers appears to be in two phases, an initial 'acute' phase of rapid healing in the first 15 to 20 days after production of the ulcers and a chronic phase during which the healing stops or the ulcers are re-exacerbated. This second phase may be analogous to the failure of the human chronic peptic ulcer to heal.

During the acute phase the wound decreased considerably in size before any epithelial regeneration occurred. The mechanism of this wound contraction is not clear but the movement of the mucosa bordering the lesion may be related to localised contraction of the outer muscle layers.
Cortisone treatment resulted initially in larger ulcers and these ulcers also failed to heal during the period of observation (90 days). Myhre (1959) noted a decrease in epithelial regeneration 15 days after production of experimental gastric defects in rats receiving cortisone. He suggested that the primary defect was a cortisone-induced depression of fibroplasia in the ulcer floor, and that the epithelial regeneration was secondary to this. Khan & Phillips (1963) reported that the decreased epithelial coverage after cortisone administration was secondary to penetration and enlargement of the ulcer.

The appearance of large ulcers after 20 days could be attributed to (a) a delay in the ulcer healing process or to (b) exacerbation or re-exacerbation of healed or nearly healed ulcers. The histological appearance lends weight to the second possibility as there is evidence of recent healing, both in the regenerating epithelium and in the ulcer floor. Also the trapping of mucosal glands deep in the ulcer floor probably resulted from repeated episodes of penetration through the ulcer floor and mucosal regeneration.

The observation that phenylbutazone had no effect on the healing of cautery-induced ulcers although producing its characteristic erosions is interesting. The implication is that acute non-penetrating erosions have no connections with full-thickness ulcer.

Guinea pigs were found to be unsuitable for this model due to the rapid healing in this species. Williams (1961) reported using guinea pigs in a study of healing of ring burns but he only examined the ulcer up to 17 days after production and was not interested in chronicity.

2.4.3. Acetic acid ulcers

The full-thickness ulcers induced by sub-serosal injection of acetic acid were similar to those induced by electrocautery. As with electrocautery it was found that in guinea pigs the healing time was relatively short compared with rats.
2.4.4. **Anti-inflammatory drugs**

Phenylbutazone and aspirin were found to produce a reproducible and high incidence of erosions in a short time after erosion formation. The methods suffer from the common disadvantage that the lesions produced are superficial erosions which heal rapidly without scarring. However these erosions may resemble the acute gastric mucosal lesions encountered in humans after ingestion of anti-inflammatory drugs (Levrat et al, 1962). and the methods may be suitable for examination of factors involved in the pathogenesis of clinical drug-induced mucosal erosions and bleeding.

2.4.5. **Corticosteroids**

Similarly corticosteroid administration gave rise to reproducible gastric damage. The lesions were extensive but did not perforate or penetrate the muscularis mucosa and thus should be regarded as erosions rather than ulcers.
CHAPTER 3

"HISTOCHEMICAL AND BIOCHEMICAL STUDIES OF GASTRIC ULCERS AND EROSIONS"

3.1. INTRODUCTION

3.1.1. The structure and function of gastric mucus

3.1.2. Histochemistry of mucus

3.2. EXPERIMENTAL

3.3. RESULTS

3.4. CONCLUSIONS
3.1. **INTRODUCTION**

3.1.1. The structure and function of gastric mucus

It is now firmly established that the principle constituents of mucus are glycoproteins, namely, conjugated proteins containing as prosthetic groups one or more heterosaccharide, usually branched, with a relatively low number of sugar residues.

In spite of much pioneering work carried out, most of our knowledge of mucosubstances has been gained in the last decade, due to the advent of such techniques as ion-exchange separation and gas-liquid chromatography.

The amino-acid compositions of a number of mucins are known, eg. submaxillary glycoproteins from several species in which threonine, serine, proline, alanine and glycine were found to constitute about 70% of the total. Relatively little is known about gastric mucus because of the difficulties involved in separation from the numerous other gastrointestinal secretions. However in the principal gastric glycoprotein obtained by Schrager (1969) serine and threonine constituted between 45 and 50% of the amino acid content and threonine, serine, proline, alanine and glycine made up between 75 and 80% of the total amino acid content.

Glycoproteins have characteristic sugar components which include D-galactose, D-mannose, D-glucose, L-fucose, D-xylose, L-arabinose, N-acetyl-D-glucosamine and the N- and O-acetyl and N-glycolyl derivatives of neuraminic acid. The gastric glycoprotein obtained from gastric aspirates and from extracts of gastric mucosae contained the above carbohydrates except mannose and glucose (Schrager & Cates, 1974). The carbohydrate side-chains were composed of four sugars: N-acetyl-galactosamine, N-acetylglucosamine, galactose and fucose showing a ratio 1:3:4:2. Superimposed on the basic structure were additional sugar residues, the blood group determinants.

The sulphate in gastric aspirates would appear not to be from gastric mucus, at least in healthy subjects, but
from saliva (Schrager & Cates, 1971; Lambert et al, 1971a) and from bronchial secretions (Roussel et al, 1972).

Studies on pig gastric mucin have shown that the non-dialysable fraction of the aqueous extract of mucosal scrapings from the cardiac region can be separated into a water-insoluble mucus gel (80% by weight) and a viscous but soluble mucus (20% by weight) (Allen & Snary, 1972). The water-soluble mucus can be separated by gel filtration into high and low molecular weight mucoprotein components A and B respectively. The amino acid analyses of mucoproteins A and B are identical and in particular they are characterised by a high content of serine, threonine and proline which together account for 42% of the total amino acids present.

Snary et al (1970) have suggested that the glycoprotein of gastric mucus consists of repeating subunits, with four of these subunits linked by disulphide bridges between cysteine residues to form mucoprotein B and that the more complex mucoprotein A consists of repeating units of mucoprotein B.

Further work is required on the molecular architecture of the complex substance 'mucus'. Only by knowledge of the structure and behaviour of the molecular components of mucus can a complete understanding of the biology of gastric mucus be acquired. The findings so far suggest that the freshly secreted product of the mucus cell is in a gelatinous form. The secretions of the neighbouring mucus cells fuse and form a continuous layer, a protective covering for the mucosa and the mucus cell may therefore be thought of as an organ producing a substance with a definite physiological function, namely, that of protecting and stabilizing the micro-environment of the mucosa.

The measurement of one or more of the individual carbohydrate fractions as an indication of the mucus content was introduced by Grossberg et al (1950) who measured the hexosamine and uronic acid present in canine gastric mucus. Various other carbohydrates have been measured and used as an indication of
mucus secretion in various studies since. For the present study, several methods were chosen for measurement of the carbohydrate content of gastric mucosal scrapings and of acid-precipitable glycoprotein as an index of mucus content.

3.1.2. Histochemistry of mucus

Mucins may be readily classified into neutral and acidic types on the basis of histochemical staining reactions. Neutral mucins consist of hexosamine and hexose units and do not have free acidic groups. Acid mucins consist of hexosamine units which may be associated with glucuronic acid, iduronic acid or sialic acid. Sulphate radicles may be present but if they are not then the carboxyl group of sialic or glucuronic acid will be the reactive acid group. Acid mucins can be further subdivided into (a) strongly acidic sulphated (b) weakly acidic sulphated (c) non-sulphated sialic acid containing (d) non-sulphated uronic acid containing and (e) non-sulphated sialidase and hyaluronidase resistant.

The techniques used in the present study for the demonstration of mucins are outlined below and in Table 3.1.

Periodic Acid-Schiff (P.A.S.) Method

Periodic acid is a very strong oxidising agent which will oxidise a vicinal diol (1,2-glycol) whether in the cis or trans form to bring about scission of a C-C bond in the pyran ring with the formation of a dialdehyde. The formation of the aldehyde results in the recolourisation of the fuchsin present in the Schiff reagent.

The acid mucopolysaccarides (hyaluronic acid, the chondroitin sulphates, etc.) all contain 1,2-glycol groups but have been shown to be P.A.S. negative, presumably due to the presence of a highly charged group in the molecule (COOH or OSO₃⁻) which interferes with the periodic acid oxidation. Although there is some controversy over sialic acids (Quintarelli et al, 1960) sialidase-labile sialomucins tend to be P.A.S. positive.
### Table 3.1
Summary of histochemical staining techniques used.

<table>
<thead>
<tr>
<th>Method</th>
<th>Chemical reaction involved</th>
<th>Histochemical result</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alcian blue at controlled pH</td>
<td>Probably formation of alcian blue complexes with carboxyls and some sulphates</td>
<td>At pH 2.5 sialomucins and weakly acid sulfomucins stain blue; the most strongly acidic sulfomucins stain weakly or not at all. At pH 1.0 weakly and strongly acidic sulfomucins stain selectively.</td>
</tr>
<tr>
<td>levels</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Periodic acid-Schiff (P.A.S.)</td>
<td>Oxidation of vicinal hydroxyls to dialdehydes by periodate and formation of coloured complexes with Schiff's reagent</td>
<td>All PS and MS containing hexoses and deoxyhexoses with vic-hydroxyl groups, i.e. periodate reactive polymers, stain red to magenta.</td>
</tr>
<tr>
<td>Alcian blue pH 2.6 - P.A.S.</td>
<td>Addition of results by single methods</td>
<td>Neutral PS and MS colour magenta; alcian blue reactive, periodate unreactive MS stain blue. Alcian blue and periodate reactive MS colour purple-blue.</td>
</tr>
<tr>
<td>Alcian blue pH 1.0 - P.A.S.</td>
<td>Addition of results by single methods</td>
<td>Sulphated MS stain blue or purple-blue, neutral and non-sulphated, periodate reactive-acid MS stain red.</td>
</tr>
</tbody>
</table>

PS = polysaccharide

MS = mucosubstance
Alcian blue

Alcian blue is a water soluble copper phthalocyanin dye of unknown structure which forms alcian blue complexes with acidic groups. Lev & Spicer (1964) used alcian blue at pH 1.0 and 2.5 to distinguish between acid and sulphated mucopolysaccarides or glycoproteins. At pH 1.0, carboxyl groups are not ionised and do not stain whereas sulphate groups are demonstrated. At pH 2.5 carboxyl groups stain well while sulphated mucins may stain poorly (Spicer & Sun, 1967).

Many other techniques for the histochemical determination of mucins are available, often using metachromatic dyes. Blocking techniques can also be used to aid identification of a particular mucin.
3.2. EXPERIMENTAL

3.2.1. Materials

Animals, conditions of housing, drugs, electrocautery apparatus, anaesthetics and materials for histochemical processing were described in Section 2.2.1.

The following solutions were prepared for histochemical staining:
(a) **Barger and De Lameter's Schiff reagent** Basic fuchsin, (George T. Gurr Ltd.) 1 g was dissolved in 400 ml of distilled water and 1 ml of thionyl chloride added. After shaking, the flask was allowed to stand for 12 hours and then 2 g of activated charcoal added, the solution shaken and immediately filtered.
(b) **Sulphite rinses** were freshly prepared by adding 7.5 ml of 1 M hydrochloric acid to 135 ml distilled water.
(c) **Alcian blue pH 2.5** consisted of 1% alcian blue (alcian blue 8GX, George T. Gurr Ltd.) in 3% acetic acid. Solution filtered before use.
(d) **Alcian blue pH 1.0** consisted of 1% alcian blue (alcian blue 8GX, George T. Gurr Ltd.) in 0.2 M HCl.

The following standards used for estimation of carbohydrates were all obtained from B.D.H. Biochemicals: glucosamine hydrochloride, L(-)fucose, galactose, mannose, N-acetylneuraminic acid, 2-deoxyribose, bovine serum albumin.

3.2.2. Treatment of animals

To evaluate the effect of ulcerogenic procedures on the histochemistry of gastric mucus, groups of rats were subjected to restraint either for 2 hours at 7-10°C or for 24 hours at 15-17°C as described in Section 2.2.3. Administration of phenylbutazone and prednisolone and the production of electrocautery ulcers were also described in the relevant sections of Chapter 2.

For the effect of stress on the carbohydrate components of gastric mucosa, two groups of 6 female rats, 150 g bodyweight were used. One group was restrained in wire-mesh at 14-17°C.
for 24 hours, while the other group served as controls. After the restraint period, the rats were sacrificed by cervical dislocation and the stomachs were excised and rated for pathology as described previously (Section 2.2.3).

Aspirin and phenylbutazone were administered to groups of 8 female rats, 150 g bodyweight at a dose of 200 mg/kg orally. One group of rats served as controls and received vehicle alone. The rats were sacrificed by cervical dislocation 6 hours after drug administration and the stomachs excised and rated as described in Section 2.2.3.

3.2.3. Preparation of tissues for histochemical and biochemical evaluation

Paraffin sections of stomach tissue were prepared as described in Section 2.2.2.

Mucosal scrapings were prepared by carefully scraping off the glandular mucosa from the underlying muscularis mucosa. The mucosal scrapings were freeze-dried overnight in an Edwards Freeze Drier Model EF 03 before weighing and homogenisation in 0.1M Tris buffer, pH 7.4. Aliquots of the homogenate were used for the various estimations.

The acid-precipitable glycoprotein fraction was obtained after homogenisation of the glandular stomach in 20 ml of 5 mM Na₂EDTA (pH 7.4) by precipitation with 10% trichloroacetic acid and 1% phosphotungstic acid. The precipitate was washed twice with water and two lipid extractions performed using chloroform/methanol (1/1, v/v). After air-drying the acid precipitable glycoproteins were weighed and samples used for the various estimations.

3.2.4. Histochemical staining

After preliminary experiments to determine suitable methods, techniques employing the periodic acid-Schiff (P.A.S.) procedure either alone or in combination with alcian blue at different pH values was used.
P.A.S. technique After removal of wax and hydration (Section 2.2.2.) the sections were oxidised for 10 minutes in 1% aqueous periodic acid followed by a wash in running water for 5 minutes and a rinse in distilled water. The sections were then placed in Schiff reagent for 30 minutes followed by 1 minute in the first sulphite rinse and 2 minutes in the second sulphite rinse. The sections were washed for 10 minutes before dehydrating, clearing and mounting in D.P.X.

Combined alcian blue - P.A.S. techniques The method is as described above except that after bringing to water the sections were stained with the appropriate alcian blue solution for 20 minutes before proceeding with the P.A.S. technique. When using the alcian blue solution at pH 1.0 the slides were blotted dry instead of rinsing in distilled water. This was to prevent the blueing of non-sulphated sialic acid containing sites on rinsing with water as it appears that the water rinse raises the pH of the slide towards neutrality and hence above the pH of the carboxyl groups, permitting the combination with cationic dye molecules. Despite the diluting effect of the water, the carboxyl-containing sites presumably have such an avidity for the dye that they react with it even during the brief water rinse (Lev & Spicer, 1964).

3.2.5 Determination of carbohydrate content of gastric mucosal scrapings and of acid-precipitable glycoproteins

In the methods described below, spectrophotometric determinations were made on a Perkin-Elmer Double Beam Spectrophotometer and Recorder.

(1) Gastric mucosal scrapings

Hexosamines An aliquot (0.25 ml) of the homogenate was diluted to 1 ml with water and hydrolysed overnight at 100°C in 2 ml 2M HCl. After cooling, the mixture was diluted to 10 ml with water and filtered and 1-3 ml of the filtrate was assayed according to the method of Boas (1953) modified by the omission of the resin separation, as preliminary experiments gave similar results.
without this step. Glucosamine hydrochloride was used as a standard.

Fucose  An aliquot (0.1 ml) of the homogenate was diluted to 1 ml with water and assayed by the method of Gibbons (1955) using L(-) fucose as a standard.

Sialic acids  An aliquot (1 ml) of the mucosal homogenate was hydrolysed in 0.1 M H₂SO₄ at 80°C for 1 hour (this procedure splits off the bound sialic acids without dehydration). The hydrolysate was freeze-dried overnight and reconstituted in 0.2 ml for the assay of sialic acids by the method of Warren (1959). N-acetylneuraminic acid was used as a standard.

It was found that there was a second absorption maximum at 533 nm due to 2-deoxyribose for which a correction had to be made since the light absorption of this material at 549 nm was considerable. This correction factor was obtained by determining the molecular extinction coefficients of N-acetylneuraminic acid (NANA) and 2-deoxyribose (2DR) and substituting them into the following equation:

\[
\mu\text{mol NANA} = \left[ \frac{E_3}{E_2E_3 - E_1E_4} \times \text{OD}_{549\text{nm}} - \frac{E_4}{E_2E_3 - E_1E_4} \times \text{OD}_{532\text{nm}} \right] \times 4.3
\]

where

- \( E_1 \) = molecular extinction coefficient of NANA at 532 nm \( \times 10^{-3} \)
- \( E_2 \) = molecular extinction coefficient of NANA at 549 nm \( \times 10^{-3} \)
- \( E_3 \) = molecular extinction coefficient of 2DR at 532 nm \( \times 10^{-3} \)
- \( E_4 \) = molecular extinction coefficient of 2DR at 549 nm \( \times 10^{-3} \)

Lower molecular extinction coefficients than quoted by Warren were found: \( E_1 = 8.5; E_2 = 20; E_3 = 48; E_4 = 28 \).

This gave \( \mu\text{mol NANA} = (0.265\text{OD}_{549} - 0.159\text{OD}_{532}) \times 10^3 \)

Protein  The assay was carried out according to Lowry et al (1951) using 0.1 ml of the mucosal homogenate diluted to 0.5 ml with water. Bovine serum albumin was used as the standard.

(2) Dried acid-precipitable glycoprotein

Hexosamines, fucose and hexoses  Approximately 20 mg of each
body mucosa sample was accurately weighed and hydrolysed overnight in 2 M HCl. After hydrolysis, the samples were filtered and made up to 5 ml with distilled water.

**Hexoses and fucose** The method of Dische and Shettles (1948) for the combined estimation of fucose and hexose was used, utilizing L(-)fucose, D-galactose and D-mannose as standards for accurate determination of wavelengths.

**Hexosamines** Samples of the hydrolysate (3 ml) were assayed according to the method of Boas (1953) described previously.

**Sialic acids** Samples of the precipitable glycoprotein (20-40 mg) were hydrolysed for 1 hour in 2 ml 0.1 M H$_2$SO$_4$ according to the method of Warren (1959) as described above.

The molecular absorbance indices and correction factor were obtained from new measurements and only slightly different values were obtained: $E_1 = 7.3$; $E_2 = 20.6$; $E_3 = 57.6$; $E_4 = 34.6$; $\mu$mol NANA = (0.2880D$_{549}$ - 0.1670D$_{532}$) x 10$^3$
3.3. RESULTS

3.3.1. Histochemical staining

The results of staining sections of rat body and pyloric epithelium with the combined alcian blue-P.A.S. techniques are shown in Table 3.2. In rat body epithelium the superficial surface epithelial cells secrete a mixture of neutral and acidic mucosubstances. In the cells lining the foveolae the secretion is predominantly neutral, while the cells lying at the base of the pits (deep foveolar cells) secrete a predominantly acidic mucosubstance. The mucous neck cells show no affinity for alcian blue and thus are presumed to secrete a wholly neutral mucosubstance. The cells at the base of the foveolae also stain with alcian blue at pH 1.0, so that it appears that there is a mixture of acidic mucosubstances – some containing carboxyl groups (stainable with alcian blue at pH 2.5 and probably sialomucins) and others with sulphate groups (stainable with alcian blue at pH 1.0). The latter are weakly acidic sulphated as they were shown in preliminary experiments to be aldehyde-fuchsin unreactive.

In rat pyloric epithelium, the surface epithelial secretions are similar to those of body epithelium, except that the ratio of neutral to acidic mucosubstances in both the superficial and foveolar cells appears to be greater. The pyloric gland cells stain blue with alcian blue at pH 2.5 and pH 1.0 indicating that non-sulphated sialic acid-containing and weakly acidic sulphated mucins are present.

The effects of the various ulcerogenic procedures are described below:

(a) Stress

After induction of stress erosions, a complete loss of mucus staining in the area of erosion was observed (Fig. 3.1). Throughout the sections the distribution of mucus material was uneven and reduction in the staining of surface epithelium, deep foveolar cells and mucus neck cells was obvious. The deficiency appeared to be greater in the mucus neck and deep
Table 3.2.
Alcian blue - P.A.S. techniques on rat gastric mucosa.

<table>
<thead>
<tr>
<th></th>
<th>Alcian blue pH 2.6</th>
<th>Alcian blue pH 1.0</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>- P.A.S.</td>
<td>- P.A.S.</td>
</tr>
<tr>
<td><strong>BODY</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Superficial epithelium</td>
<td>4 P</td>
<td>4 R</td>
</tr>
<tr>
<td>Foveolar walls</td>
<td>4 R - P</td>
<td>4 R</td>
</tr>
<tr>
<td>Deep in foveolae</td>
<td>3 P - B</td>
<td>3 P</td>
</tr>
<tr>
<td>Mucous neck cells</td>
<td>3 R</td>
<td>4 R</td>
</tr>
<tr>
<td><strong>PYLORUS</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Superficial epithelium</td>
<td>4 R - P</td>
<td>4 R</td>
</tr>
<tr>
<td>Foveolar walls</td>
<td>4 R</td>
<td>4 R</td>
</tr>
<tr>
<td>Deep in foveolae</td>
<td>3 P</td>
<td>3 R - P</td>
</tr>
<tr>
<td>Pyloric gland cells</td>
<td>3 B</td>
<td>2 - 3B</td>
</tr>
</tbody>
</table>

R = Red;  P = Purple;  B = Blue;  0 = absent;  
1 = very weak;  2 = weak;  3 = medium;  4 = strong;
Fig. 3.1

Stress erosion in body mucosa of rat (alcian blue pH 2.5 — P.A.S., x 200).

Fig. 3.2

Stress erosion after 7 days healing (P.A.S., x 200).
foveolar cells and appeared to affect P.A.S. positive mucosubstances more than alcianophilic ones. The mucous cells appeared flattened and the lumen of the pits dilated. In the pyloric region, alcian blue staining appeared to be increased after stress (Fig. 3.3). The lumen of the glands appeared to be dilated and filled with alcian blue positive material.

Examination of the stomachs of stressed rats revealed that 7 days after recovery the eroded area had been recovered with epithelial tissue. The regenerated epithelium was of a different pattern and showed a stronger mucus staining than adjacent 'normal' mucosa which in some cases was very poorly stained, especially the surface epithelium. The regenerated epithelium differed from normal mucosa in an important respect - all the cells throughout the length of the gland showed, to a greater or lesser extent, positive staining for mucus and no parietal or peptic cells were present (Fig. 3.2). After 14 days recovery the regenerated epithelium showed only a slightly different pattern of glands from normal but again mucus-secreting cells were in the place of parietal and peptic cells.

(b) Prednisolone

The pattern of mucus staining after treatment with prednisolone was very similar to that observed after stress. Both in areas where erosions had occurred and in non-eroded areas there was complete absence of staining. In other areas superficial epithelium was well stained but deep foveolar and mucus neck cells were poorly stained. At one erosion a single layer of cells had started to migrate across the necrotic floor. These cells did not stain positive for mucus.

Examination of stomachs after prednisolone administration revealed that the lesions had been recovered with epithelium in 5-10 days. At this stage the regenerated epithelium appeared as simple downgrowths with good mucus staining. No parietal cells or basophilic peptic cells were observed but instead the cells throughout the glands secreted mucus. A similar picture presented at 15 days but after 30 days the sections appeared normal.
Antrum of rat after restraint stress (alcian blue pH 1.0 — P.A.S., x 500).
(c) **Phenylbutazone**

After phenylbutazone administration changes in mucus staining were less marked than after stress or prednisolone but the mucus neck cells produced slightly less intense staining and occasional areas were devoid of staining.

(d) **Electrocautery**

Electrocautery produced regions of cell death corresponding to complete absence of P.A.S. staining. In the initial stages some glands remained relatively intact, although they displayed a decreased amount of P.A.S.-positive material.

After 10 days, epithelial regeneration was observed in most ulcers although in some cases this amounted to only a single layer one cell deep, migrating across the floor (Fig.3.4). In others the regenerating epithelium was more complex but a common feature was that all but the most advanced cells displayed P.A.S. staining. In the epithelium adjacent to the ulcer the parietal and peptic cells were replaced by mucus-producing cells (Fig.3.5). The cells in the bases of the glands secreted a neutral mucosubstance, while the cells halfway up the gland secreted a mixture of sialic-acid containing and sulphated mucosubstances. The regenerating epithelium secreted a mixture of neutral and acidic, non-sulphated mucus at first but in more complex epithelium sulphated mucosubstances staining with alcian blue at pH 1.0 were also secreted. Alcian blue staining of the floor of the ulcers varied from almost strong to nil but no correlation could be made between the histological appearances (haematoxylin & eosin) and the intensity of staining.

It was also observed that the mucous neck cells of undamaged mucosa were in general, poorly stained and that the epithelial secretions were stained to a greater extent with P.A.S. and less with alcian blue pH 2.6 than normal indicating a greater proportion of neutral to acidic mucoproteins.

3.3.2. **Carbohydrate content of mucosal scrapings and acid-precipitable glycoproteins**

(1) **Effect of stress on the carbohydrate content of mucosal scrapings**

The main effect observed after 24 hours restraint
Fig. 3.4

Migrating epithelium after electrocautery (alcian blue pH 1.0 — P.A.S.) x 400.

Fig. 3.5

Regenerating epithelium 15 days after electrocautery (alcain blue pH 2.5 — P.A.S., x 200).
was a significant reduction in the total amount of freeze-dried mucosal scrapings with an associated reduction in the total hexosamines, sialic acids, fucose and protein (Table 3.3). However the tissue concentration was significantly altered only in the case of protein, which showed a significant elevation from the control value even though the total amount of protein was reduced compared with the control rats (Table 3.4).

(2) Effect of aspirin and phenylbutazone on the carbohydrate content of acid-precipitable glycoprotein

The carbohydrate content of the acid-precipitable glycoprotein after administration of aspirin and phenylbutazone is shown in Table 3.5. A significant reduction in the hexose content was observed in the phenylbutazone-treated group and a similar trend is apparent in the aspirin group although the reduction in the latter only just reaches statistical significance at the 5% level.

No differences in the content of hexosamines or sialic acid was observed.

If separate consideration is given to rats which developed erosions, then further interesting differences can be seen (Table 3.6). In the aspirin group the changes in hexose and fucose content are significant in the rats with erosions. The changes are much greater in rats with erosions than in those without.

In the phenylbutazone treated group a significant rise in hexosamine content was observed in rats without erosions while hexosamine values in the rats with erosions did not differ from controls. The reduction in hexose content was greater in rats with erosions than in those without but was significantly different from controls in both groups. The fucose content appeared similar in rats with or without erosions and, although perhaps slightly raised, failed to reach significance at the 5% level.
Table 3.3

Effect of restraint stress on the total scrapings of rat gastric mucosa.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. of Rats</th>
<th>Freeze-dried Scrapings (mg)</th>
<th>Protein (mg)</th>
<th>Fucose (µmol)</th>
<th>Hexosamines (µmol)</th>
<th>Sialic acids (µmol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>6</td>
<td>68 ± 4</td>
<td>8.8 ± 0.3</td>
<td>2.6 ± 0.7</td>
<td>4.0 ± 0.5</td>
<td>0.078 ± 0.004</td>
</tr>
<tr>
<td>Restraint</td>
<td>6</td>
<td>47 ± 2&lt;sup&gt;(3)&lt;/sup&gt;</td>
<td>7.1 ± 0.3&lt;sup&gt;(2)&lt;/sup&gt;</td>
<td>2.2 ± 0.2</td>
<td>2.7 ± 0.2&lt;sup&gt;(1)&lt;/sup&gt;</td>
<td>0.057 ± 0.005&lt;sup&gt;(1)&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Rats were restrained for 24h at 14 - 17°C.

<sup>(1)</sup>P < 0.05;  <sup>(2)</sup>P < 0.01;  <sup>(3)</sup>P < 0.001; (Students t test).
Table 3.4

Effects of restraint stress on protein and carbohydrate content of rat gastric mucosal scrapings.

<table>
<thead>
<tr>
<th>Treatment of Rats</th>
<th>No.</th>
<th>Protein (µg/mg tissue)</th>
<th>Carbohydrate content (nmol/mg tissue)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>6</td>
<td>129 ± 5</td>
<td>312 ± 96</td>
<td>60 ± 9</td>
</tr>
<tr>
<td>Restraint</td>
<td>6</td>
<td>153 ± 3(2)</td>
<td>305 ± 27</td>
<td>60 ± 6</td>
</tr>
</tbody>
</table>

Rats were restrained for 24h at 14 - 17°C.

(2) P < 0.01 (Students t test).
<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. of Rats</th>
<th>Carbohydrate content, nmol/mg glycoprotein</th>
<th>hexosamines</th>
<th>fucose</th>
<th>sialic acids</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>8</td>
<td>33 ± 2</td>
<td>61 ± 13</td>
<td>24 ± 2</td>
<td>0.40 ± 0.03</td>
</tr>
<tr>
<td>Aspirin 200 mg/kg orally</td>
<td>8</td>
<td>33 ± 2</td>
<td>29 ± 8(1)</td>
<td>21 ± 2</td>
<td>0.39 ± 0.01</td>
</tr>
<tr>
<td>Phenylbutazone 200 mg/kg orally</td>
<td>8</td>
<td>37 ± 2</td>
<td>18 ± 4(1)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Rats were sacrificed 6h after drug administration.

Note: *P < 0.05 (Students t test).*
Table 3.6.

Effect of aspirin and phenylbutazone on the carbohydrate content of gastric glycoproteins in rats with and without erosions.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Erosions Present</th>
<th>No. of Rats</th>
<th>Carbohydrate content ((\text{nmol} / \text{mg glycoprotein}))</th>
<th>hexosamines</th>
<th>hexoses</th>
<th>fucose</th>
<th>sialic acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>No</td>
<td>8</td>
<td>(33 \pm 2)</td>
<td>(61 \pm 12)</td>
<td>(14 \pm 3)</td>
<td></td>
<td>(0.40 \pm 0.03)</td>
</tr>
<tr>
<td>Aspirin 200mg/kg orally</td>
<td>No</td>
<td>3</td>
<td>(30 \pm 6)</td>
<td>(46 \pm 17)</td>
<td>(20 \pm 4)</td>
<td></td>
<td>(0.42 \pm 0.03)</td>
</tr>
<tr>
<td></td>
<td>Yes</td>
<td>5</td>
<td>(35 \pm 3)</td>
<td>(19 + 4(2))</td>
<td>(26 + 2(2))</td>
<td></td>
<td>(0.39 \pm 0.01)</td>
</tr>
<tr>
<td>Phenylbutazone 200mg/kg orally</td>
<td>No</td>
<td>4</td>
<td>(41 \pm 1(2))</td>
<td>(21 \pm 6(1))</td>
<td>(21 \pm 2)</td>
<td></td>
<td>(0.39 \pm 0.01)</td>
</tr>
<tr>
<td></td>
<td>Yes</td>
<td>4</td>
<td>(34 \pm 3)</td>
<td>(15 \pm 3(2))</td>
<td>(21 \pm 3)</td>
<td></td>
<td>(0.38 \pm 0.01)</td>
</tr>
</tbody>
</table>

Rats were sacrificed 6h after drug administration

\((1)_{P \leq 0.05}; \quad (2)_{P \leq 0.01}\) (Students t test).
3.4. CONCLUSIONS

3.4.1. Histochemical staining

A decrease in P.A.S. staining after restraint stress has previously been reported by Ludwig & Lipkin (1969) and Lambert et al (1971b). The present results confirm this, showing an overall decrease in the mucosa of sulphated, sialic acid-containing and neutral mucosubstances. The zone which regenerates the mucosa, incorporating the deep foveolar and mucous neck cells appeared to be affected the most, and this may be important in the generation of the lesions. The observation that the neutral mucosubstances were reduced to a greater extent than the acidic mucosubstances may also be important as in the past particular attention has been paid to the sulphated mucosubstances because these molecules inhibit the proteolytic activity of pepsin (Anderson & Baillie, 1967).

The appearance of the mucus-secreting cells also suggested intensive secretory activity because of the flattened mucous cells and dilated pits. This was noticed especially in the antral region of the rat stomach after stress where the lumen of the glands appeared to be dilated and filled with weakly sulphated and acidic mucosubstances. In general, alcian blue staining of the rat antrum after stress appeared to be increased which may explain why the antrum is more resistant to erosion formation. This apparent increase in mucus synthesis may be a prelude to a following decrease in synthesis as observed in the body of the stomach. In any case it appears that the more general reduction in mucus may be attributable to both a reduction in mucus synthesis and an increase in secretion.

Histochemical staining of the lesions after 7 and 14 days recovery showed the pattern of formation of new glands by mucin-producing cells with parietal and zymogen cells conspicuous by their absence. It has been shown that not only are the new glands deficient in parietal and zymogen cells but that the glands in the immediate vicinity of the mucosal lesion seem to lose
these cells and come to contain more mucin-producing cells than usual (McMinn & Johnson, 1963). There is no histological evidence to suggest that zymogen or parietal cells are lost by extrusion, and it is usually held that embryologically they develop from the mucous neck variety (McMinn & Johnson, 1963). It seems therefore the zymogen and parietal cells de-differentiate into mucin-producing cells.

The findings after prednisolone administration of damage to the gastric epithelium and a decrease in mucus content agree with previous findings of corticosteroids in man, dog and rat. In contrast to this Lev et al (1970) found that small doses of prednisolone actually stimulated mucus production by gastric surface epithelial cells and they questioned the concept that the ulcerogenic effect of steroids is related to their ability to depress formation of mucus. In the present study, however, no increase in mucus was seen and non-eroded areas showed a decrease in staining, indicating that the alteration in mucus probably preceded erosion formation.

As with stress erosions, epithelial regeneration was quick, and in fact was already in progress during treatment as evidenced by the finding of cell migration across the floor of one erosion. The lack of mucus in these migrating cells is indicative of their regenerative state. The healing process was similar to that observed with stress erosions with new gland formation by mucus cells and the absence of parietal and zymogen cells. However these cells were found at a later stage of healing suggesting that they differentiate from mucus cells between two and four weeks after erosion formation. This agrees with the findings of Myhre (1956) and Hunt (1958) who found parietal and zymogen cells in the rat at three to five weeks after injury.

Although the changes occurring after phenylbutazone administration were not so marked as after stress or prednisolone, again the mucous neck cells appeared to be affected most. The areas devoid of mucus staining are probably those in an immediate pre-ulcerogenic stage.
P.A.S. staining of the stomach gives a good indication of cellular damage after electrocautery with normal staining in the undamaged mucosa to complete absence of staining where extensive damage had occurred. The decrease in mucus appeared to precede the complete destruction of the cells.

The epithelium regeneration from epithelium advancing from the margin of the ulcer and formation of new glands from downgrowths of new epithelium into the granulation tissue were discussed in a previous section (Chapter 2) but it is of interest to note that the migrating epithelial cells quickly attain the ability to secrete mucus as all but the most advanced cells were seen to be P.A.S. positive. Differences from normal epithelium were confirmed by P.A.S. - alcian blue staining showing that the glands at the margin of the lesion lose their complement of parietal and zymogen cells, presumably by dedifferentiation, and become capable of secreting mucin. The changes in the pattern of mucus secretion are difficult to interpret but it is possible that the de-differentiation occurring in the glands adjacent to the ulcer allows greater secretion of mucus for protection of the regenerating epithelium. The migrating cells themselves are quick to develop the ability to secrete mucus probably for the same reason but the significance of the changing pattern of mucus secretion, with sulphated mucins secreted in more complex epithelium is not known.

Lack of correlation of intensity of staining of the ulcer floor with histological appearance is not surprising because alcian blue at pH 1.0 stains mucosubstances of epithelial, rather than connective tissue origin. For examination of the degree of formation of connective tissue it would be necessary to use alcian blue at pH 0.5

3.4.2. Carbohydrate content of mucosal secretions and acid-precipitable glycoprotein

Several reports have previously shown that carbohydrate components of the mucus of gastric mucosa are modified after
fasting, restraint stress and ulcerogenic drugs (Robert et al., 1963; Ludwig & Lipkin, 1969; Lambert et al., 1971b; Robert & Bezamis, 1963; Menguy & Masters, 1965; Menguy & Desailletts, 1967). The observed changes in mucosal scrapings in the stressed rats in the present study probably reflect the loss of mucous cells observed histologically. The tissue content of the various carbohydrates was not significantly altered after stress and this may reflect the fact that although decreased mucus staining was observed histologically in some areas of the mucosa, in other areas there was an increase in mucus secretion. However, the protein content of the mucosa was raised after restraint stress even though the total protein was decreased along with total carbohydrates. This may indicate that as well as a general loss of mucosubstance, the mucus contained a relatively greater amount of protein and less carbohydrate. It is important to study the changes in mucus in relation to the morphology of the mucous cells as the mucus secretion is not uniform throughout the mucosa and thus misleading interpretations can be made from biochemical data alone.

The changes in the carbohydrate-composition of the acid-precipitable glycoproteins after administration of aspirin and phenylbutazone suggest that modifications in the composition of mucus as well as in the quantity of mucus secreted may be important. It has been stated that the property of mucus of resisting enzymic hydrolysis may be related to its carbohydrate moieties (Menguy & Masters, 1965). The precise effects of the changes in fucose, hexose, and hexosamine content observed in the present experiments are not known but it is reasonable to assume that the quantity of secreted mucus will have altered, possibly in such a way as to render it less efficient as a protective barrier. The changes in mucous secretion determined biochemically are in agreement with the histochemical picture after alcian blue and P.A.S. staining.

Previous studies on the effect of aspirin on mucous secretion in rats and dogs showed that the sialic acid concentration
of secreted mucoprotein was altered (Menguy & Masters, 1965). The authors discussed the importance of this in view of the fact that since sialic acid occupies a terminal position on the carbohydrate prosthetic groups of mucous glycoprotein, then it would be important in steric hindrance of proteolytic enzymes attacking the peptide bonds of the protein core.

The histochemical and biochemical methods used to study gastric mucus and its alteration as mucosal damage occurs has given us important information. Much further work remains to be done, particularly on the molecular structure of mucus and its biosynthesis within the mucus cells.
CHAPTER 4

"EFFECTS OF FASTING, STRESS AND DRUGS ON GASTRIC GLYCOPROTEIN SYNTHESIS"

4.1. INTRODUCTION

4.1.1. Biosynthesis of glycoproteins

4.1.2. Role of glycoproteins in cell adhesion

4.1.3. Radioactively labelled precursor incorporation

4.2. EXPERIMENTAL

4.3. RESULTS

4.4. CONCLUSIONS
4.1. **INTRODUCTION**

In a previous chapter the structure and function of mucus was reviewed. It is proposed in this chapter to review the synthesis of the most important constituents of mucus, the glycoproteins.

4.1.1. **Biosynthesis of glycoproteins**

(a) **Glycopeptide bonds**

Glycosylation of the polypeptide chain of a glycoprotein usually involves the amino acids serine, threonine, asparagine, hydroxylysine and hydroxyproline. Apart from asparagine, they all contain hydroxyl groups but there is no other obvious structural similarity among the amino acid residues which are glycosylated. However each codon for L-asparagine (AAU and AAC) can give rise by single base substitutions to codons for L-serine (AGU and AGC respectively), L-threonine (ACU and ACC) and L-lysine (AAA and AAG) (Marshall, 1972). Several distinct types of glycopeptide bond have been described but all the mucins studied so far contain carbohydrate units linked to serine and threonine by N-acetylgalactosamine residues. The sugar components which make up these units include sialic acid, fucose, galactose, N-acetylgalactosamine and N-acetylglucosamine (Spiro, 1970).

(b) **Subcellular sites of glycosylation**

The study of glycoprotein biosynthesis has taken two major experimental approaches: (i) the use of intact cells and (ii) the study of glycosyltransferases in cell free systems, and from these a model of glycoprotein synthesis has been developed (Schachter & Roden, 1972).

Kinetic data from work on intact cells is interpreted as showing that the sugars are mainly added in a stepwise fashion while the peptides move from rough endoplasmic reticulum
to smooth endoplasmic reticulum and then to the Golgi region where they are concentrated and packaged for secretion. There is evidence that a small amount of N-acetylglucosamine may be attached to the peptide which is still nascent on the ribosomes but the precise stage at which it becomes incorporated into the chain remains to be determined (Marshall, 1972). Studies of cell-free systems have shown various glycosyltransferases in high concentrations in the Golgi apparatus (Schachter et al, 1970).

A picture has evolved in which the glycosyltransferases function to transfer activated sugars from their nucleotide derivatives to appropriate receptors (Spiro, 1970). The synthesis of carbohydrate units of defined structure is determined by the specificity of these transferases, which is directed towards the sugar nucleotide as well as the acceptor. The enzymes are specific for the protein-carbohydrate acceptor and the sugar nucleotide and in this way structures with defined sequence, branching, position of the intersaccaride linkages and the nature of the glycopeptide bonds are obtained. Typical reactions are shown below:

(i) \[ \text{CMP-NAN} + \text{HO-R-protein} \rightarrow \text{NAN-C-R-protein} + \text{CMP} \]

\[ R = \text{galactose, N-acetylgalactosamine} \]
\[ \text{NAN} = \text{N-acetylneuraminic acid} \]

(ii) \[ \text{XDP-S} + \text{HO-R-protein} \rightarrow \text{S-O-R-protein} + \text{XDP} \]

\[ X = \text{uridine, guanine} \]
\[ S = \text{galactose, glucosamine, N-acetylg glucosamine, N-acetylgalactosamine, xylose, fucose, mannose} \]
\[ R = \text{ saccharide or hydroxyaminoacid} \]

(c) **Regulation of glycoprotein biosynthesis**

Attachment of the carbohydrate is post-ribosomal and therefore not under direct genetic control as is protein synthesis. Synthesis of oligosaccharides appears to be controlled
by the substrate specificities of these transferases and thus every oligosaccharide prosthetic group is assembled by the concerted action of a multi-glycosyltransferase system. Oligosaccharide sequences may not only be controlled by the specificities of glycosyltransferases but also by the physical arrangement of the multi-glycosyltransferase systems on the membrane.

The stages of carbohydrate attachment are thus logical points for the physiological or pharmacological control of glycoprotein biosynthesis and may also be important in pathological processes (Spiro, 1970).

(d) Catabolism of glycoproteins

It appears that glycosidases present in the lysosomes can act together to degrade the carbohydrate units of glycoproteins (Aronson & de Duve, 1968; Mahadevan et al, 1969). However to date little is known about the part they play in the overall regulation of glycoprotein synthesis and degradation.

4.1.2. Role of glycoproteins in cell adhesion

The presence of carbohydrates on the surface of mammalian cells has been demonstrated by a variety of methods, including histochemical studies (Rambourg & Leblond, 1967) and the interaction of antibodies with surface antigens (Watkins, 1966). It appears that surface glycoproteins and/or glycolipids are involved with cell adhesion and surface glycosyltransferases have been implicated in the process (Roseman, 1970).

It seems logical therefore that gastric mucosal cells may depend on the synthesis of glycoproteins to maintain cell adhesiveness and thus the gastric mucosal barrier and optimum cell turnover rates.
4.1.3. Radioactively labelled precursor incorporation

Coffey et al (1964) were the first to study intestinal glycoprotein synthesis with the use of a radioactive precursor, L-\( ^{14}C \) fucose, along with a mucosal fractionation procedure. Forstner (1970) described events following the intraperitoneal injection of \( ^{14}C \) glucosamine where the radioactivity is incorporated first into microsomes, and later in glycoproteins attached to the microvillus membrane and in luminal secretions. Autoradiographic evidence following pulse labelling with other polysaccharide precursors is also consistent with a model in which intestinal glycoproteins are synthesised in the microsomes and Golgi complex and then transferred to the microvillus membrane and to the lumen as secreted mucus (Neutra & Leblond, 1966a, b; Ito & Revel, 1968).

Lukie & Forstner (1972a) developed an in vitro system of measuring \( ^{14}C \) glucosamine incorporation by intestinal slices. Their results indicated that glycoprotein synthesis could be studied in isolated intestinal slices for periods of at least 3 hours. This method was investigated by Shillingford, Lindup & Parke (1974) and modified for measurement of glycoprotein synthesis in pieces of rat stomach. These authors investigated a series of labelled hexoses and amino-sugars and found that N-acetyl-D-\( ^{3}H \) glucosamine gave the best rates of incorporation. The labelled hexosamine was thus chosen for the present work using methodology closely based on the methods of Lukie & Forstner (1972a) and Shillingford et al (1974).
4.2. EXPERIMENTAL

4.2.1. Materials

Animals, conditions of housing etc., drugs were described in Section 2.2.1.

N-acetyl-D-[1-3H]glucosamine (4Ci/mmol) and L-[3-3H]serine (15Ci/mmol) were obtained from the Radiochemical Centre, Amersham. Scintillator chemicals were obtained from the Packard Instrument Co., all other chemicals and solvents being obtained from R.D.H. or other usual laboratory suppliers.

4.2.2. Treatment of animals

Rats were restrained for 24 hours at normal room temperature of 22°C or for 24 hours or 6 hours at a lower temperature (17°C) as described in Section 2.2.3. The rats were not fasted prior to the restraint period but had no access to food during restraint and therefore groups of rats fasted for the same length of time and at the same temperature as the restrained rats were included to determine the effects of fasting alone.

Phenylbutazone, aspirin and prednisolone were suspended in 0.1% aqueous Tween 80 and administered orally to rats via a stainless steel tube at a dose of 200 mg/kg (0.5 ml/100 g bodyweight). (-)-Adrenaline bitartrate was dissolved in saline and administered intraperitoneally to rats at a dose of 2 mg/kg (0.5 ml/100 g bodyweight). The animals were killed 6 hours after a single administration in most experiments but in one experiment rats were dosed orally daily for 7 days before sacrifice.

After sacrifice the stomachs were excised and washed in ice-cold saline. The stomachs were examined and rated for pathology as described in Section 2.2.3.

4.2.3. Assessment of glycoprotein synthesis

The rates of incorporation of N-acetyl-[3H]glucosamine and L-[3H]serine into gastric mucosal glycoproteins were measured
by modifications of the method of Lukie & Forstner (1972a). Circular pieces of glandular stomach were removed using a cork borer to ensure similar sample sizes. Initially experiments were carried out to compare the rates of incorporation obtained with tissue samples of different size (either 7 mm or 14 mm diameter) or from different parts of the glandular stomach (either body or antrum). Most of the subsequent experiments were carried out on a piece of tissue 14 mm diameter from the body mucosa.

Incubations were carried out at 37°C in a Mickle shaking incubator (60 rev/min). The samples were pre-incubated for 10 minutes in a modified Krebs Medium I (Lukie & Forstner, 1972a) before addition of 1μCi of either N-acetyl-[\(^3\)H]glucosamine or L-[\(^3\)H]serine in 0.1 ml saline. The final incubation medium volume was 2 ml containing: NaCl (236 μmol); KCl (11.2 μmol); CaCl₂ (6.0 μmol); KH₂PO₄ (2.8 μmol); MgSO₄ (2.8 μmol); NaHCO₃ (38.6 μmol); L-glutamine (16 μmol).

Incubations were carried out for 2.5 hours in most experiments but up to 4 hours to investigate the relationship between the rate of incorporation and time of incubation. The samples were gassed with a mixture of 95% O₂ and 5% CO₂ every 20 minutes. Incubations were terminated by draining the medium and washing the samples twice with 5.0 ml of ice-cold saline. The sample tissues were then homogenised in 20 ml of 5 mM disodium ethylenediamine tetraacetate (EDTA; pH 7.4). Glycoproteins were precipitated overnight at 4°C with 10% trichloroacetic acid and 1% phosphotungstic acid. The acid-soluble supernatant was discarded and the precipitate washed twice with distilled water followed by two lipid extractions with chloroform/methanol (1:1, v/v). After air-drying, the precipitate was weighed and solubilised overnight in 2 ml of 0.5M NaOH. The samples were acidified before adding a 1 ml aliquot to 10 ml scintillant consisting of 3.3 g/l 2,5-diphenyl-oxazole and 0.2 g/l 1,4-di-2-(4 methyl-5-phenyl-oxazolyl benzene in Triton X-100/toluene (1:2, v/v). The tritium content was determined in a Packard Tricarb Model 3320 liquid scintillation spectrometer.
4.3. **RESULTS**

4.3.1. **Effect of site of tissue sample**

Antral tissue was found to have approximately twice the rate of incorporation of N-acetylglucosamine than samples from the corpus (Table 4.1).

4.3.2. **Effect of size of tissue sample and time of incubation**

The rate of incorporation remained linear over 3 hours and there was little difference between the different-sized tissues (Fig. 4.1).

4.3.3. **Effects of fasting, stress and drugs**

The effects of fasting, restraint and cold and combinations of these treatments are given in Table 4.2. It was found that temperature markedly affected the degree of mucosal damage in the restraint experiments, the incidence and severity of erosions being far greater in the restraint plus cold group than with restraint alone for 24 hours. A reduction of the rate of incorporation of N-acetylglucosamine was also observed in the restrained rats but this was similar for the restraint and restraint plus cold groups with no correlation between the extent of mucosal damage and the reduction in rate of incorporation. Fasting for 24 hours produced a reduction in the rate of incorporation of N-acetylglucosamine similar to that observed with 24 hours restraint but without causing gastric mucosal damage. The effect of fasting plus cold for 24 hours on the rate of incorporation of N-acetylglucosamine was slightly greater than that of fasting alone but not significantly so and again, no mucosal damage was observed. With a shorter experimental period of 6 hours, cold alone or fasting plus cold produced no mucosal damage whereas restraint plus cold for 6 hours resulted in erosions in 3 out of 6 rats. The rate of incorporation of N-acetylglucosamine was not significantly reduced after cold.
Table 4.1

Effect of site of tissue sample on rate of incorporation of N-acetylglucosamine into rat gastric mucosal glycoprotein.

<table>
<thead>
<tr>
<th>Tissue site</th>
<th>No. of Rats</th>
<th>Incorporation of N-acetylglucosamine mmol x 10^{-12}/mg glycoprotein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body</td>
<td>9</td>
<td>126 ± 22</td>
</tr>
<tr>
<td>Antrum</td>
<td>8</td>
<td>278 ± 50 (P&lt;0.01)</td>
</tr>
</tbody>
</table>

Portions of tissue were incubated with 1.0 μCi N-acetyl-(^{3}H)glucosamine for 2.5 hours. Probability value (P) of significance of difference is also given (Student's 't' test).
Fig. 4.1.

Effect of tissue size and time of incubation on the rate of incorporation of N-acetylglucosamine into rat gastric glycoproteins

Portions of corpus tissue were incubated for varying times. Values shown are means for groups of 12 rats ± S.E. mean.
Table 4.2

The effect of fasting, restraint and cold on the rate of incorporation of N-acetylglucosamine into rat gastric glycoproteins.

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of Rats</th>
<th>% with erosions</th>
<th>Mean erosion index</th>
<th>Incorporation of N-acetyl glucosamine mmol x 10^{-12}/mg glycoprotein</th>
<th>% of Control value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>9</td>
<td>0</td>
<td>0</td>
<td>171 ± 23</td>
<td>100</td>
</tr>
<tr>
<td>fasted 24h</td>
<td>4</td>
<td>0</td>
<td>0</td>
<td>81 ± 27(^{(1)})</td>
<td>47</td>
</tr>
<tr>
<td>restrained 24h</td>
<td>4</td>
<td>75</td>
<td>1.5</td>
<td>61 ± 19(^{(2)})</td>
<td>35</td>
</tr>
<tr>
<td>cold 24h</td>
<td>10</td>
<td>0</td>
<td>0</td>
<td>211 ± 36</td>
<td>124</td>
</tr>
<tr>
<td>fasted + cold 24h</td>
<td>4</td>
<td>0</td>
<td>0</td>
<td>57 ± 15(^{(2)})</td>
<td>33</td>
</tr>
<tr>
<td>restrained + cold 24h</td>
<td>4</td>
<td>100</td>
<td>5.3</td>
<td>82 ± 34(^{(1)})</td>
<td>48</td>
</tr>
<tr>
<td>cold 6h</td>
<td>6</td>
<td>0</td>
<td>0</td>
<td>159 ± 47</td>
<td>92</td>
</tr>
<tr>
<td>fasted + cold 6h</td>
<td>6</td>
<td>0</td>
<td>0</td>
<td>125 ± 24</td>
<td>73</td>
</tr>
<tr>
<td>restrained + cold 6h</td>
<td>6</td>
<td>50</td>
<td>1.8</td>
<td>72 ± 17(^{(2)})</td>
<td>42</td>
</tr>
</tbody>
</table>

Portions of body mucosa were incubated with 1.0 μCi N-acetyl-(\(^3\)H)glucosamine for 2.5 hours. Value shown are means for the number of rats (n) ± s.e. mean.

\(^{(1)}p<0.05; \quad (2)p<0.01\) (Students t test).
or fasting plus cold for 6 hours, but was reduced after restraint plus cold for 6 hours.

In a separate series of experiments, the results of which are given in Table 4.3, restraint plus cold for 6 hours, aspirin (200 mg/kg orally) and phenylbutazone (200 mg/kg orally) all resulted in acute gastric mucosal erosions in 37-60% of the treated rats. Adrenaline (2 mg/kg i.p.) produced severe sub-mucosal haemorrhage with no sign of discrete erosion or ulceration and the rats receiving prednisolone (200 mg/kg orally) showed no gross gastric pathology. The rate of incorporation of N-acetylglucosamine was significantly reduced after restraint plus cold for 6 hours and after phenylbutazone administration, but not after aspirin, adrenaline or prednisolone. In fact, in the prednisolone treated group, 3 out of 5 rats had markedly low rates of incorporation with 2 animals giving values in the normal range. The reduction failed to attain statistical significance owing to the large variation within the group.

If separate consideration is given to the rats which developed erosions (Table 4.4) then restraint plus cold for 6 hours, phenylbutazone and aspirin all gave significant reductions in the rate of incorporation of N-acetylglucosamine in rats with erosions. In the treated rats which failed to develop erosions, restraint plus cold for 6 hours produced a small but non-significant reduction in N-acetylglucosamine incorporation, while aspirin increased the incorporation but again not significantly so. A significant reduction in the rate of incorporation of the labelled hexosamine in rats without erosions was seen only in the group receiving phenylbutazone.

In contrast to their effects on the rate of incorporation of N-acetylglucosamine, aspirin and phenylbutazone appeared to increase the rate of incorporation of L-serine into rat gastric mucosal glycoproteins although the phenylbutazone value just failed to attain statistical significance at the 5% level using a two-tailed Student's t test (Table 4.5).
Table 4.3.

The effect of various procedures on the rate of incorporation of N-acetylglucosamine into gastric glycoproteins.

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of Rates</th>
<th>% with erosions</th>
<th>Mean index</th>
<th>Incorporation of N-acetyl glucosamine (mmol x 10^{-12}/mg glycoprotein)</th>
<th>% of Control value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>22</td>
<td>0</td>
<td>0</td>
<td>166 ± 17</td>
<td>100</td>
</tr>
<tr>
<td>Restraint + cold 6h</td>
<td>11</td>
<td>55</td>
<td>1.9</td>
<td>100 ± 17(1)</td>
<td>61</td>
</tr>
<tr>
<td>Phenylbutazone 200mg/kg orally</td>
<td>11</td>
<td>37</td>
<td>0.9</td>
<td>102 ± 14(2)</td>
<td>62</td>
</tr>
<tr>
<td>Aspirin 200mg/kg orally</td>
<td>10</td>
<td>60</td>
<td>1.4</td>
<td>133 ± 25</td>
<td>80</td>
</tr>
<tr>
<td>Adrenaline 2mg/kg i.p.</td>
<td>6</td>
<td>0</td>
<td>0</td>
<td>180 ± 36</td>
<td>109</td>
</tr>
<tr>
<td>Prednisolone 200mg/kg orally</td>
<td>5</td>
<td>0</td>
<td>0</td>
<td>93 ± 35</td>
<td>57</td>
</tr>
</tbody>
</table>

Rats were sacrificed after 6h restraint or 6h after drug administration. Portions of body mucosa were incubated with 1.0 μCi of N-acetyl-(3H)glucosamine for 2.5h. 

\( P < 0.05; \quad P < 0.01 \quad \text{(Students t test).} \)
Table 4.4

Inhibition of the rate of incorporation of N-acetylglucosamine into gastric glycoproteins in rats with and without gastric erosions.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Erosions Present</th>
<th>No. of Rats</th>
<th>Incorporation of N-acetylglucosamine mmol \times 10^{-12}/mg glycoprotein</th>
<th>% of control value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>No</td>
<td>22</td>
<td>166 ± 17</td>
<td>100</td>
</tr>
<tr>
<td>Restraint + cold 6h</td>
<td>No</td>
<td>5</td>
<td>127 ± 30</td>
<td>77</td>
</tr>
<tr>
<td></td>
<td>Yes</td>
<td>6</td>
<td>78 ± 17(3)</td>
<td>47</td>
</tr>
<tr>
<td>Phenylbutazone 200mg/kg orally</td>
<td>No</td>
<td>7</td>
<td>108 ± 19(1)</td>
<td>66</td>
</tr>
<tr>
<td></td>
<td>Yes</td>
<td>4</td>
<td>91 ± 18(2)</td>
<td>55</td>
</tr>
<tr>
<td>Aspirin 200mg/kg orally</td>
<td>No</td>
<td>4</td>
<td>202 ± 36</td>
<td>122</td>
</tr>
<tr>
<td></td>
<td>Yes</td>
<td>6</td>
<td>87 ± 17(2)</td>
<td>52</td>
</tr>
</tbody>
</table>

Rats were sacrificed after 6h restraint or 6h after drug administration. Portions of body mucosa were incubated with 1.0 \mu Ci of N-acetyl-(^3H)glucosamine for 2.5h.

\( (1)_{P < 0.05}; \quad (2)_{P < 0.01}; \quad (3)_{P < 0.001} \) (Students t test).
Table 4.5.

Effect of aspirin and phenylbutazone on the rate of incorporation of L-serine into rat gastric glycoproteins.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. of Rats</th>
<th>% with erosions</th>
<th>Mean erosion index</th>
<th>Incorporation of L-serine mmol x 10^{-12}/mg glycoprotein</th>
<th>% of Control Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>12</td>
<td>0</td>
<td>0</td>
<td>17 ± 2</td>
<td>100</td>
</tr>
<tr>
<td>aspirin (200mg/kg orally)</td>
<td>12</td>
<td>50</td>
<td>1.4</td>
<td>25 ± 2^{(2)}</td>
<td>52</td>
</tr>
<tr>
<td>phenylbutazone (200mg/kg orally)</td>
<td>12</td>
<td>58</td>
<td>2.1</td>
<td>22 ± 1^{(a)}</td>
<td>78</td>
</tr>
</tbody>
</table>

Rats were sacrificed 6h after drug administration. Portions of body mucosa were incubated with 1.0 μCi L-\(^3\)H-serine for 2.5h.

^{(a)} P < 0.1, \(^{(2)} P < 0.01\) (Students t test).
If separate consideration is given to rats with and without erosions present after treatment, then no significant differences are found between rats with and without erosions in the same treatment group. However the rise of the rate of incorporation of L-serine is more marked in the aspirin-treated rats with erosions than in those without while in the phenylbutazone-treated group the converse applies (Table 4.6).

4.3.4. **Effect of sub-acute administration of drugs**

After 7 days oral administration of aspirin, phenylbutazone and prednisolone (all at 200 mg/kg daily) no significant differences in the rate of incorporation of N-acetylglucosamine were found. This was despite the fact that erosions were present in the rats from all treated groups (Table 4.7).
Table 4.6.

Rate of incorporation of L-serine into rat gastric glycoproteins in rats with and without erosions.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Erosions present</th>
<th>No. of Rats</th>
<th>Incorporation of L-(^{3})H serine mmol \times 10^{-12}/mg glycoprotein</th>
<th>% of Control value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>No</td>
<td>12</td>
<td>17 ± 2</td>
<td>100</td>
</tr>
<tr>
<td>Aspirin 200mg/kg orally</td>
<td>No</td>
<td>6</td>
<td>24 ± 2</td>
<td>137</td>
</tr>
<tr>
<td></td>
<td>Yes</td>
<td>6</td>
<td>27 ± 3(^{(1)})</td>
<td>159</td>
</tr>
<tr>
<td>Phenylbutazone 200mg/kg orally</td>
<td>No</td>
<td>7</td>
<td>25 ± 2(^{(1)})</td>
<td>142</td>
</tr>
<tr>
<td></td>
<td>Yes</td>
<td>5</td>
<td>20 ± 2</td>
<td>119</td>
</tr>
</tbody>
</table>

Rats were sacrificed 6h after drug administration. Portions of body mucosa were incubated with 1.0 \(\mu\)Ci of L-\(^{3}\)H serine for 2.5h.

\(^{(1)}\) \(p < 0.05\), (Students t test).
### Table 4.7

The effect of sub-acute (7 days) administration of drugs on the rate of incorporation of N-acetylglucosamine into rat gastric glycoproteins

<table>
<thead>
<tr>
<th>Group</th>
<th>No of Rats</th>
<th>% with erosions</th>
<th>Mean erosion index</th>
<th>Incorporation of N-acetylglucosamine mmol x 10^-12/mg glycoprotein</th>
<th>% of Control value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>6</td>
<td>0</td>
<td>0</td>
<td>50 ± 13</td>
<td>100</td>
</tr>
<tr>
<td>Aspirin 200mg/kg orally</td>
<td>6</td>
<td>100</td>
<td>4.3</td>
<td>42 ± 7</td>
<td>84</td>
</tr>
<tr>
<td>Phenylbutazone 200mg/kg orally</td>
<td>6</td>
<td>67</td>
<td>2.0</td>
<td>43 ± 5</td>
<td>88</td>
</tr>
<tr>
<td>Prednisolone 200mg/kg orally</td>
<td>6</td>
<td>67</td>
<td>2.7</td>
<td>57 ± 6</td>
<td>114</td>
</tr>
</tbody>
</table>

Rats were sacrificed 24h after the final drug administration. Portions of body mucosa were incubated with 1.0 μCi of N-acetyl-(³H)glucosamine for 2.5h. None of the values of N-acetylglucosamine incorporation are significantly different from controls.
4.4. CONCLUSIONS

4.4.1. Effect of site of tissue sample

The rate of incorporation of N-acetylglucosamine appears to be twice as great in the antrum as the body region of the stomach. This is probably because the body contains, in addition to mucus cells, parietal and peptic cells and thus the glycoprotein precipitation procedure gives more glycoprotein and protein not associated with mucus.

4.4.2. Effect of size of tissue sample and time of incubation

The results indicate that the system is satisfactory over a three hour period with tissue sizes of either 7 mm or 14 mm diameter.

4.4.3. Effects of fasting, stress and drugs

In the present study, there are three theoretical possibilities regarding a relationship between erosion formation and the observed inhibition of glycoprotein synthesis. First, erosion formation and the inhibition of glycoprotein synthesis are not related: second, impaired glycoprotein synthesis may be a direct result of mucosal cell damage: and third, mucosal pathology may be a consequence of impaired glycoprotein synthesis.

The first possibility is seen in fasting for 24 hours which causes an inhibition in the rate of incorporation of N-acetylglucosamine into gastric glycoproteins without causing gastric mucosal erosions. The incorporation also appeared to be lowered in some rats after prednisolone treatment, although no mucosal damage occurred, while adrenaline resulted in severe gastric pathology without any reduction in glycoprotein synthesis. However, it is known that more prolonged fasting (Robert et al, 1963) does lead to mucosal damage and may be a necessary prerequisite or even the cause of the damage. Also, the nature of
the adrenaline-induced gastric damage was markedly different from that after treatment with the other agents used in this study. No discrete erosions or ulcerations were present but diffuse sub-mucosal haemorrhage affecting large areas of the stomach was observed. Thus, the primary effect of adrenaline appears to be on the blood vessels, causing vasoconstriction with no immediate effect on glycoprotein biosynthesis, although more prolonged treatment may lead to an inhibition of synthesis possibly through reduction of the mucosal blood supply.

The second possibility, that impaired glycoprotein synthesis is a result of mucosal damage is unlikely as first, the reduction in N-acetylglucosamine incorporation can occur when no mucosal damage is present (eg. after 24 hours fasting and in phenylbutazone-treated rats which failed to develop erosions) and second, when mucosal damage does occur, the reduction of the rate of incorporation of N-acetylglucosamine is not related to the intensity of the damage (eg. restraint and restraint plus cold for 24 hours, Table 4.2).

The third possibility, that mucosal damage is a consequence of impaired glycoprotein synthesis may apply as erosion formation after restraint plus cold stress, aspirin or phenylbutazone was found to be associated with a reduction in the rate of incorporation of N-acetylglucosamine into rat gastric glycoproteins. Some of the changes in the rate of incorporation of N-acetylglucosamine occurred in treated rats which had not developed erosions, suggesting that they not only accompany, but actually precede, erosion formation.

Decreases in the quantity, and carbohydrate content, of secreted mucus in rat and dog have previously been reported after administration of aspirin (Menguy & Masters, 1965), phenylbutazone (Menguy & Desbaillets, 1967) and prednisolone (Robert & Nezamis, 1963). Decreases in the glycoprotein content of rat and guinea pig gastric mucosa have also been shown after restraint stress (Ludwig & Lipkin, 1969; Lambert et al, 1971b).

Thus it is possible that one mode of action of stress and drugs in causing gastric erosions is inhibition of
the glycosylation of the mucus glycoprotein and hence a decreased synthesis of glycoprotein or the synthesis of a modified glycoprotein or both. In a recent study, Sander et al (1975) demonstrated that immobilization of rats in a cold environment inhibited the activity of glucosamine synthetase in the gastric mucosa, an effect leading to reduced synthesis of hexosamine-containing compounds. A decreased synthesis of glycoproteins as measured by radioactive glucose uptake by sheep colonic mucosal scrapings in the presence of aspirin in vitro has previously been found (Kent & Allen, 1968). Lukie & Forstner (1972b) have also shown inhibition of \( \text{L-[}^{14}\text{C}] \) glucosamine incorporation into rat intestinal glycoproteins by sodium salicylate in vitro. These in vitro studies support the present results obtained with in vitro incubation after drug administration in vivo.

Decreased synthesis of glycoprotein or synthesis of a modified glycoprotein may not only be important in relation to the quantity or quality of secreted mucus. Decreased cell adhesiveness as a result of inhibition of glycoprotein biosynthesis may also lead to increased cell loss and in such conditions the integrity of the mucosa will not be maintained (Section 4.1.2.). It is of interest to note that, as well as their effect on mucus described, effects on cell turnover associated with erosion formation have been observed after restraint stress (Kim et al, 1970), and salicylates, phenylbutazone and corticosteroids (Max & Menguy, 1969, 1970).

4.4.4. Effect of sub-acute administration of drugs

The lack of effect on glycoprotein synthesis even though gastric damage was present may be explained in two ways:

1) The synthesis of the glycoproteins has adapted to the ulcerogenic agents in such a way that after repeated administration a reduction in the incorporation rate is no longer seen. St. John et al (1973) showed that the ulceration produced by a single dose of aspirin was greater than that produced for 3, 14, 28 and 56 days showing an altered response to a specific effect of aspirin. This altered response may also be related to an increase of the mucosal
detoxifying capacity as shown by Hietanen (1975) who found an increased gastric mucosal UDP-glucuronyltransferase activity after sub-chronic administration of aspirin.

2) As the rats were sacrificed 24 hours after the final administration of the drugs it is possible that the glycoprotein synthesis recovered to normal levels in this time. The processes of mucosal regeneration in the rat are very fast as evidenced by the healing of acute mucosal erosions.
5.1. INTRODUCTION

5.1.1. Prostaglandins and the gut

5.1.2. Cyclic nucleotides and the gut

5.1.3. Interrelationships between prostaglandins and cyclic AMP

5.1.4. Effects of fasting and glycaemia on the stomach

5.2. EXPERIMENTAL

5.3. RESULTS

5.4. CONCLUSIONS
5.1. INTRODUCTION

5.1.1. Prostaglandins and the gut

Prostaglandins are cyclohexenoic acids and are related to certain natural poly-unsaturated fatty acids such as arachidonic acid. They are present in the gastrointestinal tract and have been implicated in the physiological and pathological processes involving the gut.

Species differences have been found in the occurrence of various prostaglandins in the gut eg. PGE₁ is the predominant prostaglandin in rat gastric mucosa (Bennet et al, 1967; Shaw & Ramwell, 1968) whereas human gastric mucosa contains mainly PGE₂ (Bennet et al, 1968).

Several prostaglandins have been found to influence three major functions of the gastrointestinal tract: gastric secretion, gastrointestinal motility and intestinal absorption and secretion. For the present purposes we are mainly interested in the effects of prostaglandins on gastric secretion and ulcer formation.

Prostaglandins of the E and A (but not of the F) type have been shown to inhibit acid secretion both in vivo and in vitro (eg. Waller, 1973; Way & Durbin, 1969). The secretion of other components of gastric secretion, ie pepsin and mucus, after PGE₁ administration was also found to be reduced in one study (Robert et al, 1968).

The prostaglandins also have a direct vasodilator action but during inhibition of acid secretion gastric blood flow is reduced probably as a result rather than the cause of the antisecretory effect (Robert, 1973; Waller, 1973). These findings, together with increased release of mucosal prostaglandins during secretory stimulation (Shaw & Ramwell, 1968) have led to the hypothesis that prostaglandins have a physiological role as negative feedback inhibitors of acid secretion and functional vasodilators in the gastric mucosa.

The mechanism of action of the prostaglandins in
inhibition of acid secretion is not clear. As cyclic AMP is a postulated intracellular mediator of gastric acid secretion, one attractive suggestion is that prostaglandins inhibit adenyl cyclase, the enzyme responsible for transforming ATP into cyclic AMP. This possibility is further discussed in Section 5.1.3.

The anti-ulcer effect of natural and synthetic prostaglandins has been demonstrated in a number of models including stress (Usardi et al., 1974), pylorus ligation (Robert et al., 1968), duodenal ulcers produced by constant infusion of various gastric secretagogues (Robert et al., 1971) and administration of indomethacin (Lippmann, 1974). The mechanism of their anti-ulcer effect is commonly attributed to inhibition of acid and pepsin secretion but proof of this is lacking and does not explain the anti-ulcer effect of PGF$_{2\alpha}$ (Usardi et al., 1974) which is a poor inhibitor of acid secretion. The action of PGF$_{2\alpha}$ may be explained on the basis of vasodilator activity but no data are available on the effects of PGF$_{2\alpha}$ on gastric mucosal circulation. However, it is well known that prostaglandins of the E and F series have different vascular activity.

In humans, prostaglandins of the E and A series have been found to reduce basal and stimulated acid secretion after intravenous but not oral administration (Waller, 1973). A recent report confirmed the failure of PGE$_2$ to act orally but its synthetic analogue 15(R)15-methyl-E$_2$-methyl ester gave marked inhibition of acid secretion (Karim et al., 1973). This PGE$_2$ analogue has also been reported to possess a powerful stimulating effect on the mucus-secreting cells of the gastric mucosa in humans (Fung et al., 1974a).

The clinical usefulness of prostaglandins in the treatment of peptic ulcer disease is not yet known. It may be surmised that potent orally active synthetic analogues of prostaglandins may compete with histamine H$_2$-receptor antagonists for the control of acid secretion. One study has suggested that a deficiency
in PGE may be the basis of gastric hypersecretion in duodenal ulcer disease (Hinsdale et al., 1974) although in another study no such deficiency was found (Tonnesen et al., 1974).

5.1.2. Cyclic nucleotides and the gut

Cyclic adenosine 3', 5'-monophosphate (cyclic AMP) is a nucleotide which functions as an intracellular second messenger mediating many of the actions of a number of hormones and also serving a role in controlling key cellular processes. It has become clear that cyclic guanosine 3', 5'-monophosphate (cyclic GMP) may play a separate and somewhat independent role as a second messenger. In recent years there has been increasing evidence to suggest that cyclic nucleotides may be involved in gastrointestinal function and their role in gastric secretion will be discussed.

In spite of in vitro and in vivo and species differences, the bulk of the evidence supports the second messenger concept for cyclic AMP in increasing gastric acid secretion in response to histamine and gastrin (Kimberg, 1974). Cyclic GMP may also be involved in the control of acid secretion (Eichhorn et al., 1974).

The mechanism of action of the cyclic nucleotides is not proven but probably involves the activation of carbonic anhydrase via a protein kinase.

The possibility that cyclic AMP might mediate the secretion of pepsinogen and/or mucin from gastric mucosal cells has not been explored but Forstner et al. (1973) have reported stimulation of intestinal glycoprotein synthesis with cyclic AMP in vitro.

5.1.3. Interrelationships between prostaglandins and cyclic AMP

There is not sufficient information to be certain whether the antisecretory effect of the prostaglandins is mediated at the level of adenyl cyclase or at a more distal step in the
cellular secretory process. PGE$_1$ has been reported to stimulate cyclase activity in gastric mucosa (Perrier & Lastner, 1969) but other observations suggest inhibition rather than stimulation (Bieck, 1972).

5.1.4. Effects of fasting and glycaemia on the stomach

Although fasting is of little interest as a major aetiological factor in peptic ulcer, it has been suggested that a critically low glucose or caloric intake may account in part for gastric lesions in some patients (Mullane et al, 1974). Fasting is known to influence experimentally-induced gastric erosions or ulcers (Weisz, 1957; Robert & Nezamis, 1958; Pfeiffer, 1970a). Conversely it has been reported that hyperglycaemia protects animals from stress ulcers (Selye & Maclean, 1944) and that patients with diabetes have a lowered incidence of duodenal ulcer (Dotevall, 1959).

The present study was carried out to investigate the effects of fasting and other procedures which affect blood glucose levels on the susceptibility of rats to aspirin-induced gastric damage. The synthesis of gastric glycoproteins was also studied as an index of mucus production and mucosal cell function.
5.2. EXPERIMENTAL

5.2.1. Materials

Animals and conditions of housing were described in Section 2.2.1.

Prostaglandins $E_1$, $E_2$, and $F_2\alpha$ tromethamine salt were the generous gift of Dr. J. Pike, Upjohn Company, Kalamazoo, Michigan. Stock solutions in ethanol (10 mg/ml) were prepared and kept at 4°C.

The sodium salts of dibutyryl cyclic AMP ($N^6, O^2$-dibutyryl adenosine 3'5'-cyclic monophosphoric acid sodium) and dibutyryl cyclic GMP ($N^2, O^2$-dibutyryl guanosine 3'5'-cyclic monophosphoric acid sodium) were obtained from the Sigma Chemical Company. Stock solutions (10 mg/ml) in 50% aqueous ethanol were prepared and kept at 4°C.

Other chemicals and drugs not described in previous sections were theophylline (B.D.H.), D-glucose (B.D.H.), insulin zinc suspension B.P. 40 U/ml (Burroughs Wellcome & Co.) and alloxan monohydrate (B.D.H.). Labelled compounds and scintillator chemicals for radioactivity measurements were described in Section 4.2.1.

5.2.2. Treatment of animals

For assessments of their effects in glycoprotein synthesis prostaglandins $E_1, E_2$, and $F_2\alpha$ and the dibutyryl analogues of cyclic AMP and cyclic GMP were dissolved in either distilled water or saline and administered orally or subcutaneously to female rats at a dose of 150 µg/kg. The solutions were prepared by taking 15 µl of the stock solutions (10 mg/ml) in ethanol and diluting in water or saline to 6.5 ml, the exact volume being adjusted such that each rat received 150 µg in 1 ml of solution. Control solutions contained an equivalent amount of ethanol or 50% aqueous ethanol alone. Theophylline was dissolved in water and administered at a dose of 100 mg/kg orally.

For assessment of their effects on aspirin-induced
mucosal damage the above drugs were administered 15 minutes before a single oral administration of aspirin (200 mg/kg) suspended in 0.1% aqueous Tween 80. Theophylline was administered at higher dose levels (200 mg/kg oral and i.p.) as the lower dose had no effect on glycoprotein synthesis. Six hours after aspirin administration, the rats were sacrificed and the stomachs assessed according to the system previously described (Section 2.2.3).

The effects of glycaemia were investigated in groups of female rats, 150 g bodyweight, treated as follows: fasting for 18 hours; fasting for 18 hours but with glucose, 50 mg/ml in the drinking water; insulin 10 U/kg subcutaneously followed by removal of food; alloxan-diabetes, induced by the subcutaneous administration of alloxan monohydrate, 100 mg/kg to fasted rats five days before the experiment and subsequent feeding ad lib.

Blood samples (0.05 ml) were taken from the tails of the rats after various procedures and blood glucose estimations carried out on a Technicon Autoanalyzer II using a glucose oxidase method (Trinder, 1969a & b). After collection of blood samples the rats were sacrificed and the stomachs removed for assessment of gastric glycoprotein synthesis.

The susceptibility of rats to aspirin-induced mucosal damage was assessed in separate groups of rats dosed orally with aspirin 200 mg/kg as described above.

5.2.3. Assessment of glycoprotein synthesis

In the studies involving in vivo administration of prostaglandins and dibutyryl analogues of the cyclic nucleotides gastric glycoprotein synthesis was assessed as described previously (Section 4.2.3.) except that the incubations were carried out with homogenised glandular gastric mucosal scrapings.

In vitro studies with the above compounds were carried out on pooled homogenates of glandular mucosal scrapings. Small volumes (around 100 μl) of the stock solutions were added to around
20 ml of glandular mucosal scrapings homogenised in Krebs Medium to give the required final concentration. Control solutions received an aliquot of ethanol or 50% aqueous ethanol alone. Aliquots (1.9 ml) of the homogenates were then incubated with 1 μCi of either N-acetyl-[3H]glucosamine or L-[3H]serine and the radioactivity incorporated into the mucosal glycoproteins determined as described previously (Section 4.2.3).

Measurement of the gastric glycoprotein synthesis in the experiments investigating the effects of glycaemia was carried out as described in Section 4.2.3.
5.3. Results

5.3.1. Effect of prostaglandins and cyclic nucleotides on gastric glycoprotein synthesis and erosion formation

All three prostaglandins inhibited the rate of incorporation of N-acetylglucosamine when added to the incubation medium (Table 5.1). There was also a tendency, not statistically significant, for a reduction in L-serine incorporation with PGE₁ and PGF₂α but not with PGE₂ which, if anything, appeared to augment L-serine incorporation slightly.

Reductions in the rate of incorporation of N-acetylglucosamine and L-serine were seen after a single oral administration of the prostaglandins (Table 5.2). The reduction just failed to reach statistical significance for PGE₂ on N-acetylglucosamine incorporation. It was also observed that the stomachs of the rats appeared contracted compared with control rats.

After in vitro incubation of mucosal homogenates with the dibutyryl analogues of cyclic AMP and cyclic GMP, a significant reduction in the rate of incorporation of N-acetylglucosamine was observed with 10⁻⁴M dibutyryl cyclic AMP (Table 5.3). The rate of incorporation of L-serine also appeared to be reduced although the reduction was not significant. Dibutyryl cyclic GMP gave no significant effects while theophylline at a concentration of 10⁻⁴M gave similar effects to dibutyryl cyclic AMP.

After in vivo administration of dibutyryl cyclic AMP, reductions in the rates of incorporation of N-acetylglucosamine and L-serine were observed (Table 5.4). The route of administration appeared to alter the effect as by the oral route only the incorporation of carbohydrate was affected whereas by the subcutaneous route both carbohydrate and amino acid incorporation were reduced. Theophylline was without effect in this experiment.

The effects of various treatments on erosion formation are shown in Table 5.5. The prostaglandin representative (PGE₁)
Table 5.1

Effect of in vitro prostaglandins on the rates of incorporation of N-acetylglucosamine and L-serine into rat gastric mucosal glycoproteins.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. of incubations</th>
<th>Rate of incorporation mmol x $10^{-12}$/mg glycoprotein/2.5h</th>
<th>N-acetylglucosamine</th>
<th>L-serine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>4</td>
<td>2374±60</td>
<td></td>
<td>457±103</td>
</tr>
<tr>
<td>PGE$_1$</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$10^{-4}$M</td>
<td>4</td>
<td>1502±172(2)</td>
<td></td>
<td>301±21</td>
</tr>
<tr>
<td>PGE$_2$</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$10^{-4}$M</td>
<td>4</td>
<td>1504±291(1)</td>
<td></td>
<td>509±53</td>
</tr>
<tr>
<td>PGF$_{2\alpha}$</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$10^{-4}$M</td>
<td>4</td>
<td>977±83(3)</td>
<td></td>
<td>341±59</td>
</tr>
</tbody>
</table>

Homogenates of body mucosa were incubated with 1.0 μCi of either N-acetyl-(3H)glucosamine or L-(3H)serine for 2.5h.

(1)$_{P}<0.05$, (2)$_{P}<0.01$, (3)$_{P}<0.001$

(Student's t test)
Table 5.2

Effect of \textit{in vivo} administration of prostaglandins on the rates of incorporation of N-acetylglucosamine and L-serine into rat gastric mucosal glycoproteins.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. of Rats</th>
<th>( \text{mmol} \times 10^{-12}/\text{mg glycoprotein/2.5h} )</th>
<th>Rate of incorporation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>5</td>
<td>( 2981 \pm 348 )</td>
<td>N-acetylglucosamine</td>
</tr>
<tr>
<td>( \text{PGE}_1 )</td>
<td>(150( \mu \text{g/kg orally} ))</td>
<td>5</td>
<td>1766 ( \pm 345 )^{(1)}</td>
</tr>
<tr>
<td></td>
<td></td>
<td>429 ( \pm 33 )</td>
<td></td>
</tr>
<tr>
<td>( \text{PGE}_2 )</td>
<td>(150( \mu \text{g/kg orally} ))</td>
<td>5</td>
<td>1853 ( \pm 544 )</td>
</tr>
<tr>
<td></td>
<td></td>
<td>327 ( \pm 23 )^{(1)}</td>
<td></td>
</tr>
<tr>
<td>( \text{PGF}_2 )</td>
<td>(150( \mu \text{g/kg orally} ))</td>
<td>5</td>
<td>1417 ( \pm 183 )^{(2)}</td>
</tr>
<tr>
<td></td>
<td></td>
<td>166 ( \pm 47 )^{(2)}</td>
<td></td>
</tr>
</tbody>
</table>

Homogenates of body mucosa were incubated with 1.0 \( \mu \text{Ci} \) of either N-acetyl-(\(^3\)H)glucosamine or L-(\(^3\)H)serine for 2.5h.

\( ^{(1)}P < 0.05; \quad ^{(2)}P < 0.01; \quad ^{(3)}P < 0.001; \) (Students t test)
Table 5.3
Effect of theophylline and the dibutyryl analogues of cyclic AMP and cyclic GMP \textit{in vitro} on the rates of incorporation of N-acetylglucosamine and L-serine into rat gastric mucosal glycoproteins.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. of incubations</th>
<th>Rate of incorporation mmol x 10^{-12}/mg glycoproteins/2.5h</th>
<th>N-acetylglucosamine</th>
<th>L-serine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>4</td>
<td></td>
<td>5020 ± 670</td>
<td>341 ± 35</td>
</tr>
<tr>
<td>Dbc AMP 10^{-4}M</td>
<td>4</td>
<td></td>
<td>2990 ± 190(1)</td>
<td>226 ± 58</td>
</tr>
<tr>
<td>Dbc GMP 10^{-4}M</td>
<td>4</td>
<td></td>
<td>4160 ± 260</td>
<td>339 ± 46</td>
</tr>
<tr>
<td>Theophylline 10^{-4}M</td>
<td>4</td>
<td></td>
<td>3090 ± 250(1)</td>
<td>252 ± 56</td>
</tr>
</tbody>
</table>

Homogenates of body mucosa were incubated with 1.0 μCi of either N-acetyl-(\textsuperscript{3}H)glucosamine or L-(\textsuperscript{3}H)serine for 2.5h.

(1) \(p < 0.05\) (Students t test).
**Table 5.4**

Effect of *in vivo* administration of theophylline and the dibutylryl analogue of cyclic AMP on the rates of incorporation of N-acetylglucosamine and L-serine into rat gastric mucosal glycoproteins.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. of Rats</th>
<th>Rate of incorporation mmol x 10^-12/mg glycoproteins/2.5h</th>
<th>N-acetylglucosamine</th>
<th>L-serine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>5</td>
<td>1066 ± 254</td>
<td>190 ± 34</td>
<td></td>
</tr>
<tr>
<td>Dbc AMP 150µg/kg orally</td>
<td>5</td>
<td>359 ± 79(1)</td>
<td>147 ± 19</td>
<td></td>
</tr>
<tr>
<td>Dbc AMP 150µg/kg orally</td>
<td>5</td>
<td>368 ± 63(1)</td>
<td>85 ± 10(1)</td>
<td></td>
</tr>
<tr>
<td>Theophylline 100mg/kg orally</td>
<td>5</td>
<td>1151 ± 410</td>
<td>190 ± 31</td>
<td></td>
</tr>
</tbody>
</table>

Homogenates of body mucosa were incubated with 1.0 µCi of either N-acetyl-(³H)glucosamine or L-(³H)serine for 2.5h.

(1) P < 0.05 (Students t test).
Table 5.5

Effect of various treatments on the susceptibility of rats to aspirin-induced gastric mucosal damage.

<table>
<thead>
<tr>
<th>Pretreatment</th>
<th>No. of Rats</th>
<th>% of rats with erosions</th>
<th>Aspirin treatment</th>
<th>Mean Erosion index</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>20</td>
<td>85</td>
<td></td>
<td>2.9</td>
</tr>
<tr>
<td>PGE, 150µg/kg orally</td>
<td>10</td>
<td>10</td>
<td></td>
<td>0.2(3)</td>
</tr>
<tr>
<td>Dbc AMP, 150 µg/kg orally</td>
<td>10</td>
<td>80</td>
<td></td>
<td>3.4</td>
</tr>
<tr>
<td>Dbc AMP, 300µg/kg orally</td>
<td>10</td>
<td>70</td>
<td></td>
<td>2.5</td>
</tr>
<tr>
<td>Dbc AMP, 150µg/kg sc</td>
<td>10</td>
<td>90</td>
<td></td>
<td>2.8</td>
</tr>
<tr>
<td>Theophylline, 200mg/kg orally</td>
<td>5</td>
<td>80</td>
<td></td>
<td>2.4</td>
</tr>
</tbody>
</table>

Rats were pretreated 15 min before single administration of aspirin, 200mg/kg orally. Five rats in the theophylline group died shortly after theophylline administration and were not included.

(3) *P* < 0.002 (Mann-Whitney 'U' test).
showed good ulcer inhibitory properties while dibutyryl cyclic AMP and theophylline had no effect on the susceptibility of rats to aspirin induced damage.

5.3.2. **Effects of glycaemia on gastric glycoprotein synthesis and erosion formation**

From the results shown in Table 5.6, it can be seen that in non-diabetic rats fasting lowered blood glucose levels, increased the incidence and severity of aspirin-induced gastric mucosal damage and also reduced gastric glycoprotein synthesis by 50%. The administration of glucose via the drinking water during the fasting period prevented the fall in blood glucose levels, reduced the incidence and severity of erosions to control levels and almost completely prevented the fall in glycoprotein synthesis.

Diabetic rats showed a similar susceptibility to aspirin and gave similar values for gastric mucosal glycoprotein synthesis as non-diabetic rats (Table 5.7). The effects of fasting in diabetic rats were similar to the effects in non-diabetic rats — increased susceptibility to aspirin-induced gastric mucosal damage and reduced rate of gastric glycoprotein synthesis although blood glucose of fasted diabetic and non-fasted controls were of the same order.

In non-diabetic rats insulin lowered blood glucose levels and glycoprotein synthesis and increased the susceptibility to erosion formation (Table 5.8). However in rats made diabetic with alloxan, the effect of insulin was to lower blood glucose with no effects on the susceptibility to aspirin or on glycoprotein synthesis (Table 5.9).
Effects of fasting and fasting plus glucose on blood glucose, susceptibility to erosions and gastric glycoprotein synthesis.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Mucosal damage</th>
<th>Glycoprotein synthesis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>% of rats with erosions</td>
<td>mean erosion index</td>
</tr>
<tr>
<td>Control</td>
<td>45</td>
<td>1.8</td>
</tr>
<tr>
<td>Fasted 18h</td>
<td>100</td>
<td>4.3 (a)</td>
</tr>
<tr>
<td>Fasted 18h plus glucose 50mg/ml in drinking water</td>
<td>45</td>
<td>2.4</td>
</tr>
</tbody>
</table>

Mucosal damage was assessed in groups of 20 rats killed 6h after a single oral administration of aspirin 200mg/kg.

Measurements of blood glucose and glycoprotein synthesis are means of groups of 6 rats ± s.e.m.

Differences from control: (a) $p < 0.002$ (Mann-Whitney 'U' test); (1) $p < 0.05$; (3) $p < 0.001$ (Students t test).
**Table 5.7**

Effects of alloxan-diabetes on blood glucose, susceptibility to erosions and gastric glycoprotein synthesis.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Mucosal damage % of rats with erosions</th>
<th>Blood glucose mean index mg/100 ml</th>
<th>N-acetylglucosamine incorporation mmol x 10^{-12}/mg glycoprotein</th>
<th>Glycoprotein synthesis % of control value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>50</td>
<td>1.9</td>
<td>102 ± 2</td>
<td>211 ± 30</td>
</tr>
<tr>
<td>Alloxan - Diabetes</td>
<td>63</td>
<td>2.0</td>
<td>372 ± 18 (3)</td>
<td>203 ± 22</td>
</tr>
<tr>
<td>Alloxan-diabetes + fasted 18h.</td>
<td>100</td>
<td>3.9 (a)</td>
<td>118 ± 2 (3)</td>
<td>69 ± 32 (2)</td>
</tr>
</tbody>
</table>

Mucosal damage was assessed in groups of 8 rats killed 6h after a single oral administration of aspirin 200mg/kg.

Measurements of blood glucose and glycoprotein synthesis are means of groups of 6 rats ± s.e.m.

Differences from control: (a) $P < 0.05$ (Mann-Whitney 'U' test)

(2) $P < 0.01$; (3) $P < 0.001$ (Students t test).
Table 5.8
Effect of insulin on blood glucose, susceptibility to erosions and gastric glycoprotein synthesis.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>% of rats with erosions</th>
<th>mean erosion index</th>
<th>mean glucose mg/100 ml</th>
<th>N-acetylglucosamine incorporation mmol x 10^{-12}/mg glycoprotein</th>
<th>% of control value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>50</td>
<td>2.0</td>
<td>112 ± 11</td>
<td>198 ± 68</td>
<td>100</td>
</tr>
<tr>
<td>Insulin 10U/kg sc</td>
<td>100</td>
<td>4.5(a)</td>
<td>20 ± 3(3)</td>
<td>37 ± 8(1)</td>
<td>19</td>
</tr>
</tbody>
</table>

Mucosal damage was assessed in groups of 10 rats killed 6h after a single oral administration of aspirin.
Measurements of blood glucose and glycoprotein synthesis are means of groups 6 rats ± s.e.m.

Differences from Control: 

(a) P < 0.02 (Mann-Whitney 'U' test)

(1) P < 0.05; (3) P < 0.001 (Students t test)
Table 5.9

Effect of insulin on blood glucose, susceptibility to erosions and gastric glycoprotein synthesis in alloxan - diabetic rats.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Mucosal damage</th>
<th>Blood</th>
<th>Glycoprotein synthesis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>% of rats with erosions</td>
<td>mean erosion index</td>
<td>glucose mg/100ml</td>
</tr>
<tr>
<td>Diabetic</td>
<td>60</td>
<td>1.5</td>
<td>331 ± 27</td>
</tr>
<tr>
<td>Diabetic + insulin 10 U/kg s.c.</td>
<td>60</td>
<td>1.7</td>
<td>92 ± 15(3)</td>
</tr>
</tbody>
</table>

Mucosal damage was assessed in groups of 10 rats killed 6h after a single oral administration of aspirin.
Measurements of blood glucose and glycoprotein synthesis are means of groups of 6 rats ± s.e.m.

Differences from control: \( P < 0.001 \) (Students t test).
5.4. CONCLUSIONS

5.4.1. Effects of prostaglandins and cyclic nucleotides

The significance of the inhibition of glycoprotein synthesis after in vitro and in vivo prostaglandins is not clear but the inhibition may be related to the general reduction in components of gastric secretion i.e. acid, pepsin and mucus, found previously (Robert et al, 1968). It appears that cyclic AMP but not cyclic GMP also inhibits glycoprotein synthesis and it is therefore possible that the prostaglandin effect is mediated via stimulation of adenyl cyclase. If this is the case both cyclic nucleotides and prostaglandins would be expected to diminish the various components of gastric secretion. However the bulk of evidence suggests that prostaglandins inhibit and cyclic AMP stimulates gastric acid secretion (Sections 5.1.1. and 5.1.2.). Both stimulation and inhibition of mucosal adenyl cyclase by prostaglandins have been reported (Section 5.1.3.) so that it is extremely difficult to present a unifying picture at the present.

The inhibition of erosions by prostaglandins occurred with doses which also inhibited glycoprotein synthesis so that some mechanism other than stimulation of mucus production must be sought. The anti-ulcer effect of prostaglandins is commonly attributed to inhibition of acid and pepsin secretion but proof of this is lacking. It has been suggested that the ulcerogenic effects of anti-inflammatory drugs may be due, at least in part to inhibition of prostaglandin biosynthesis (Main & Whittle, 1975) but there appears to be poor correlation between efficacy in inhibiting prostaglandin synthesis and the ability to elicit mucosal erosions suggesting that the inhibition of prostaglandin synthesis is not the primary mechanism through which drugs erode the mucosa (Collier, 1974).

The results obtained with the dibutyryl analogue of cyclic AMP and with the phosphodiesterase inhibitor, theophylline showed no effects on erosion formation and therefore it would appear that the cyclic nucleotides do not play a major role in mucosal integrity.
5.4.2. **Effects of glycaemia**

The results show that hypoglycaemia may be a factor in susceptibility of rats to aspirin-induced gastric mucosal damage. Menguy et al (1974) found virtually no glycogen stores in the rat gastric mucosa so that the tissue depends upon a continuous supply of glucose for energy and for synthesis of the carbohydrate moieties incorporated into the mucoproteins of mucus. Acute glucose deprivation would therefore be expected to reduce the anaerobic glycolytic flux and hence energy-producing capacity of the tissue and thus impair epithelial cell function and mucus production, rendering the mucosa more susceptible to injury.

Other mechanisms may also be involved in the increased susceptibility to aspirin-induced damage produced by hypoglycaemia, e.g. hypoglycaemia stimulates gastric secretion (French et al, 1953) and consequently may favour the formation of erosions. Ragnotti & Aletti (1975) found that starvation caused the removal of ribosomes from the endoplasmic reticulum in the gastrointestinal tract and the liver. When ribosomes leave the endoplasmic reticulum, they continue to synthesize polypeptides in the cytosol, but the synthesis of glycoproteins is abated, and this may be the mechanism of the reduced rate of glycoprotein synthesis found.

It seems that the susceptibility to erosion formation and gastric glycoprotein synthesis in alloxan-diabetic rats are dependent to some extent on factors other than blood glucose levels. It may be that insulin plays a part in regulating the transfer of glucose across the epithelial cell membrane, contrary to the findings of Jolly & Cooke (1974) in oral mucosa or that removal of insulin causes decreased cellular utilization of glucose due to depression of glucose phosphorylation. Although the correlation between blood glucose levels and erosion formation is not apparent in alloxan-diabetic rats, gastric glycoprotein synthesis and erosion formation still appear to be related.
CHAPTER 6

"EFFECTS OF CARBENOXOLONE SODIUM AND ANALOGUES ON EXPERIMENTAL EROSIONS AND ON GASTRIC GLYCOPROTEIN SYNTHESIS"

6.1. INTRODUCTION

6.1.1. Carbenoxolone sodium

6.1.2. Use of the ferret to study carbenoxolone sodium

6.1.3. Analogues of carbenoxolone used in the present study

6.2. EXPERIMENTAL

6.3. RESULTS

6.4. CONCLUSIONS
6.1. INTRODUCTION

6.1.1. Carbenoxolone sodium

Carbenoxolone sodium is a triterpene, the di-sodium salt of 3-O-[β-carboxypropionyl -11-oxo-18β-olean-12-en-30-oic acid] and is synthesized from glycyrrhetinic acid, the aglycone of glycyrrhizic acid. This last compound is obtained from liquorice root.

Carefully controlled clinical trials carried out by Dr. Richard Doll and colleagues at the Central Middlesex Hospital first demonstrated a clear beneficial effect of carbenoxolone in healing of gastric ulcer (Doll et al, 1962) and these original observations have been confirmed in many clinical trials since (Sircus, 1972; Avery Jones, 1975). Side effects such as oedema, hypertension and hypokalaemia occur with carbenoxolone therapy but these are easily controlled if proper care and attention is given (Sircus, 1972).

Early trials with carbenoxolone failed to demonstrate its efficacy in patients with duodenal ulcer. This was thought to be due to the fact that carbenoxolone exerts its effects mainly by direct local action and absorption by the stomach prevented it from reaching the duodenal mucosa in sufficient amounts. A gelatine capsule was therefore devised to deliver carbenoxolone directly into the duodenum, thus by-passing the stomach. The results with this preparation of carbenoxolone have shown that it induces earlier healing of duodenal ulcer (Amure, 1970; Hunt, 1975).

The precise mode of action of carbenoxolone is unknown but in an investigation by Montgomery (1969), no effects on gastric acid secretion or motility were seen, suggesting that carbenoxolone mainly acts by strengthening the mucosal defensive forces against acid-pepsin digestion. The effects of carbenoxolone which contribute to the increased defence include increased mucus production (Goodier et al, 1967; Dean, 1968), which may be due to an increased rate of synthesis of glycoproteins in the gastric mucosa (Shillingford et
al, 1974). Domschke et al (1972) have shown that the N-acetylneuraminic acid content of gastric juice is reduced in patients with gastric ulcer and increased after carbenoxolone treatment. Lipkin (1970) showed that carbenoxolone decreased the rate of turnover of epithelial cells by prolonging their life cycle and thus led to a population of more mature cells better able to synthesize and secrete mucus. These results were confirmed in man by Croft (1973) using the gastric DNA loss as a measure of cell loss. Other activities of carbenoxolone which may be involved in its ulcer-healing properties are its anti-peptic activity (Roberts & Taylor, 1973; Birnbaum & Karmeli, 1975; Walker & Taylor, 1975) and its prevention of back diffusion of hydrogen ions (Cross et al, 1972; Thompson et al, 1975). A recent observation that carbenoxolone interferes with prostaglandin metabolism is interesting but the implications of this are not yet clear (Peskar et al, 1976).

6.1.2. Use of the ferret to study carbenoxolone sodium

The ferret would seem to be a useful species for gastroenterological study as its gastrointestinal tract is similar in many respects to that of man. Pfeiffer (1970b) examined the surface topography of the normal ferret and human gastric mucosa using a scanning electron microscope and concluded that the gastric surface topography is almost identical at the cellular level in man and ferret.

The ferret is also particularly useful for examination of the action of carbenoxolone as carbenoxolone is not hydrolysed in the ferret stomach in contrast to the rat (Shillingford, 1975). Thus the metabolism of carbenoxolone in man and ferret is similar.

6.1.3. Analogues of carbenoxolone used in present study

Several analogues of carbenoxolone with a prostaglandin-like side chain were felt to be of interest in view of the known effects of prostaglandins on the gut and these were examined for their effects on rat gastric glycoprotein synthesis (BX 494,
Another analogue, BX 505A was also investigated as it showed interesting inhibition of proteolytic activity in tests carried out by Dr. W. Taylor.

Cicloxolone sodium is a potential successor to carbenoxolone sodium in the treatment of gastrointestinal ulceration. Cicloxolone is chemically related to carbenoxolone and has been shown in various tests to be pharmacologically as active or more active than carbenoxolone.

The formulae of these compounds are shown along with that of carbenoxolone in figures 6.1 and 6.2.
Fig. 6.1

Structure of carbenoxolone sodium and ciclooxolone sodium

Carbenoxolone sodium

Ciclooxolone sodium
Fig. 6.2

Structure of some analogues of carbenoxolone sodium

\[
\begin{align*}
&\text{Ex 532A} \\
&\text{Ex 494} \\
&\text{Ex 505A}
\end{align*}
\]
6.2. EXPERIMENTAL

6.2.1. Materials

Carbenoxolone sodium (3-0 β-carboxypropionyl-11-oxo-18β-olean-12-en-30-oic acid disodium salt), and the following analogues were prepared by the Chemistry Department, Biorex Laboratories Ltd: cicloxolone sodium (3β-hydroxy-11-oxo-18β-olean-12-en-30-oic acid hydrogen cis-1,2-cyclohexanedicarboxylate disodium salt); BX 494 (2-carboxybutyrloxymethyl-18α-glycyrrhetic acid disodium salt); BX 505A (3,4 seco-18β-olean-4(23) 12 dien-3,28 dioic acid disodium salt); BX 532A (2-carboxy hexanoyloxymethyl-18α-glycyrrhetic acid disodium salt); BX 533A (2-carboxycyclohexylcarboxy)methyl-18α-glycyrrhetic acid disodium salt. Drugs were administered in solution in distilled water, drinking water or 0.9% NaCl.

Animals used were male and female Wistar rats as described previously (Section 2.2.1.). Male albino ferrets weighing approximately 2 kg each were obtained from a single supplier (A.V.Roe, Little Fakenham, Nr. Thetford, Norfolk). The ferrets were housed singly in wooden cages under the usual laboratory conditions (Section 2.2.1.) and fed on a diet of meat and milk.

The following materials were used in the electron microscopy studies: 25% glutaraldehyde (George T.Gurr Ltd.); 2% osmium tetroxide (B.D.H.); s-collidine (Koch Light); isopentane (B.D.H.); liquid nitrogen (British Oxygen Co. Ltd.). All other materials were described in previous sections.

6.2.2. Treatment of animals

For evaluation of their effects on stress erosions carbenoxolone and cicloxolone were administered to groups of 10 female rats (150 g bodyweight) at a dose of 60 mg/kg i.p., a third group serving as vehicle controls (1 ml 0.9% NaCl i.p.). Two hours after drug administration the rats were restrained
according to the method described previously (Section 2.2.3) and placed in a cold environment (12-14°C) for 3 hours. The rats were then sacrificed and the mucosal damage assessed (Section 2.2.3).

After production of electrocautery ulcers in groups of female rats weighing over 200 g (Section 2.2.4), drugs were administered for 10 days at a dose of approximately 20 mg/kg daily, via the drinking water. After treatment the rats were sacrificed and the ulceration assessed as described previously.

In the assessment of effects on glycoprotein synthesis, groups of male and female rats were treated for 7 days with carbenoxolone, cicloxxolone or various analogues at a dose of 50 mg/kg daily, half the dose being administered by stomach tube and half via the drinking water.

In the experiment using ferrets two groups of four ferrets were treated with carbenoxolone in the drinking water (50 mg/kg daily), and another two groups served as controls. The effects of acute aspirin administration were investigated in one control and one carbenoxolone pre-treated group. Aspirin was dissolved in 0.1 M HCl and administered to ferrets by oral stomach tube at a dose of 50 mg/kg (5 ml/ferret).

### 6.2.3. Assessment of glycoprotein synthesis

In the rat studies the method was as described previously (4.2.3) except that incubations were carried out using homogenised gastric mucosal scrapings with 2.5 μCi of N-acetyl-[3H]glucosamine. The medium contained 'cold' N-acetylglucosamine (25 mM) also.

In the ferret studies, the incubations were carried out for four hours using homogenised gastric mucosal scrapings with 1 μCi of either N-acetyl[3H]glucosamine or L-[3H]serine.

### 6.2.4. Scanning electron microscopy

After treatment of the ferrets as described above, pieces of stomach were excised and fixed at room temperature.
for 20-30 minutes in a formaldehyde-glutaraldehyde fixative described by Karnovsky (1965). The tissues were then cut into small blocks and fixation continued for 2-5 hours. The tissue blocks were then washed overnight in cold 0.1M phosphate buffer, pH 7.4-7.6, before post fixing in cold 1.33% osmium tetroxide buffered with s-collidine for 2 hours.

The tissues were rapidly frozen in isopentane cooled to around -160°C in liquid nitrogen and then dried for 4 hours at -40°C in an Edwards-Pearse dryer. After freeze-drying, the samples were attached to aluminium mounting stubs and electrical continuity of specimen with stub accomplished with silver paint. The sample blocks were then coated with gold-palladium in an Edwards Coating Unit.

Scanning electron microscopy was carried out on an S4 Stereoscan Scanning Electron Microscope (Cambridge Scientific Instruments Limited) operating at 20 kV with the specimen usually tilted at 30°.
6.3. RESULTS

Both carbenoxolone and ciclooxolone showed good activity in prevention of gastric erosions induced by restraint plus cold (Table 6.1). Carbenoxolone reduced by about half the incidence and severity of erosion formation while ciclooxolone completely prevented the appearance of erosions.

Both carbenoxolone and ciclooxolone also showed significant acceleration of the healing of electrocautery ulcers (Table 6.2). There was no significant difference between treatments but ciclooxolone showed a greater reduction in ulcer size in spite of the drug intake being lower.

The in vitro rate of incorporation of N-acetylglucosamine into gastric mucosal glycoproteins in male rats was significantly enhanced by pre-treatment with carbenoxolone and ciclooxolone (Table 6.3). The effect of carbenoxolone was greater than that of ciclooxolone in this respect although there was no statistically significant difference between the treatments.

Comparative studies carried out with carbenoxolone and other analogues on female rats showed that the females do not appear to be as sensitive as males to the stimulatory effect of carbenoxolone on glycoprotein synthesis (Table 6.4). However these results did show inhibition of glycoprotein synthesis with BX 532A, no effect with BX 494 and BX 533A and stimulation with BX 505A.

In the ferret study with carbenoxolone and aspirin it was established that serum levels of carbenoxolone comparable to those found in humans were obtained after 7 days pre-treatment via the drinking water. The levels were (mean ± s.e.m.) 26±5 and 29 ± 11 respectively for the two carbenoxolone-treated groups of ferrets.

Aspirin administration to the ferrets produced haemorrhagic linear erosions and the damage was assessed according to the number of erosions and their total length. Carbenoxolone pretreatment appeared to protect against aspirin-induced damage as there was
Table 6.1

Effect of carbenoxolone and cicloxolone on the erosions induced by 3 hours restraint plus cold.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. of Rats</th>
<th>No. of deaths</th>
<th>% of rats with erosions</th>
<th>Mean erosion index</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>10</td>
<td>1</td>
<td>67</td>
<td>2.7</td>
</tr>
<tr>
<td>Carbenoxolone 60mg/kg i.P.</td>
<td>10</td>
<td>0</td>
<td>30</td>
<td>1.3</td>
</tr>
<tr>
<td>Cicloxolone 60mg/kg i.P.</td>
<td>10</td>
<td>1</td>
<td>0</td>
<td>0(1)</td>
</tr>
</tbody>
</table>

(1) \( p < 0.02 \) (Mann-Whitney 'U' test)
Table 6.2

Effect of 10 day carbenoxolone and cicloxolone treatment on the healing of electrocautery ulcers in the rat.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. of rats</th>
<th>Mean Daily dose mg/kg</th>
<th>Mean ulcer index mm² ± s.e.m.</th>
<th>% of control value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>30</td>
<td>0</td>
<td>14.1 ± 1.2</td>
<td>100</td>
</tr>
<tr>
<td>Carbenoxolone</td>
<td>7</td>
<td>20</td>
<td>7.4 ± 1.5&lt;sup&gt;(2)&lt;/sup&gt;</td>
<td>52</td>
</tr>
<tr>
<td>Cicloxolone</td>
<td>6</td>
<td>15</td>
<td>5.0 ± 1.6&lt;sup&gt;(3)&lt;/sup&gt;</td>
<td>35</td>
</tr>
</tbody>
</table>

<sup>(2)</sup> P < 0.01 (Students t test)

<sup>(3)</sup> P < 0.001 (Students t test)
Table 6.3

Effect of 7 day carbenoxolone and ciclooxolone treatment (50mg/kg daily) on the rate of incorporation of N-acetylglucosamine into rat gastric mucosal glycoproteins.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. of Rats</th>
<th>Incorporation of N-acetylglucosamine (nmol/mg glycoprotein)</th>
<th>% of control value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>11</td>
<td>19.3 ± 3.2</td>
<td>100</td>
</tr>
<tr>
<td>Ciclooxolone</td>
<td>12</td>
<td>32.6 ± 3.8(^{1})</td>
<td>168</td>
</tr>
<tr>
<td>Carbenoxolone</td>
<td>12</td>
<td>42.9 ± 8.1(^{1})</td>
<td>222</td>
</tr>
</tbody>
</table>

Drugs were administered orally via the drinking water. Homogenates of mucosal scrapings were incubated with 2.5 μCi N-acetyl-(\(^{3}\)H)glucosamine for 4h.

\(^{1}\) P < 0.05 (Students t test)
Table 6.4

Effect of 7 day oral administration of carbenoxolone and analogues (50mg/kg daily) on the rate of incorporation of N-acetylglucosamine into rat gastric mucosal glycoproteins.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. of Rats</th>
<th>Incorporation of N-acetylglucosamine</th>
<th>% of control value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>12</td>
<td>6.7 ± 0.6</td>
<td>100</td>
</tr>
<tr>
<td>BX 494</td>
<td>6</td>
<td>5.8 ± 0.4</td>
<td>87</td>
</tr>
<tr>
<td>BX 505A</td>
<td>6</td>
<td>8.6 ± 1.5</td>
<td>128</td>
</tr>
<tr>
<td>BX 532A</td>
<td>6</td>
<td>3.0 ± 1.2(1)</td>
<td>45</td>
</tr>
<tr>
<td>BX 533A</td>
<td>6</td>
<td>6.9 ± 0.9</td>
<td>103</td>
</tr>
<tr>
<td>Carbenoxolone</td>
<td>12</td>
<td>9.5 ± 1.1(1)</td>
<td>142</td>
</tr>
</tbody>
</table>

Drugs were administered daily, 25mg/kg by stomach tube and 25mg/kg (approx.) via drinking water. Homogenates of mucosal scrapings were incubated with 2.5 μCi N-acetyl-(3H)glucosamine for 4h.

(1)p < 0.05 (Students t test).
a significant reduction in the number of erosions and a reduction in total length of erosions which just failed to reach statistical significance at the 5% level (Table 6.5).

Carbenoxolone significantly increased the rate of incorporation of N-acetylglucosamine into body mucosal glycoproteins (Table 6.6). Aspirin administration produced no significant change in incorporation rate or modified the stimulatory effect of carbenoxolone.

The rate of incorporation of L-serine into body mucosal glycoproteins also appeared to be increased after carbenoxolone treatment although the effect was not so great and just failed to reach statistical significance. Aspirin increased the rate of incorporation of L-serine and this effect was not modified by carbenoxolone treatment.

Scanning electron microscopy of the ferret stomachs after aspirin and carbenoxolone treatment revealed a loss of structure after aspirin, an effect which was partially prevented by carbenoxolone pretreatment. It appears however that detailed conclusions are not possible owing to artefactual damage of the mucosa by the drying procedure.
Table 6.5
Effect of 7 day carbenoxolone treatment (25mg/kg daily) on the mucosal damage induced by a single oral administration of aspirin (50mg/kg).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No of ferrets</th>
<th>Mean No. of erosions</th>
<th>Mean Total length of erosions mm.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aspirin</td>
<td>4</td>
<td>18.5</td>
<td>12.4</td>
</tr>
<tr>
<td>Carbenoxolone plus aspirin</td>
<td>4</td>
<td>7.8 (2)</td>
<td>3.0 (1)</td>
</tr>
</tbody>
</table>

Carbenoxolone was administered via the drinking water. Aspirin was suspended in 0.1N HCl and administered by stomach tube (5ml/ferret).

(1) \( P = 0.057 \) (Mann-Whitney 'U' test)

(2) \( P = 0.029 \) (Mann-Whitney 'U' test)
Table 6.6

Effect of carbenoxolone and aspirin on the rate of incorporation of N-acetylglucosamine and L-serine into ferret gastric mucosal glycoproteins.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. of Ferrets</th>
<th>Incorporation into mucosal glycoproteins mol x 10^{-12}/mg glycoprotein</th>
<th>N-acetylglucosamine</th>
<th>L-serine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>4</td>
<td></td>
<td>127 ± 30</td>
<td>51 ± 17</td>
</tr>
<tr>
<td>Carbenoxolone</td>
<td>4</td>
<td></td>
<td>1020 ± 248(2)</td>
<td>111 ± 19</td>
</tr>
<tr>
<td>Aspirin</td>
<td>4</td>
<td></td>
<td>404 ± 231</td>
<td>257 ± 80(1)</td>
</tr>
<tr>
<td>Carbenoxolone plus aspirin</td>
<td>4</td>
<td></td>
<td>1567 ± 375(2)</td>
<td>205 ± 87</td>
</tr>
</tbody>
</table>

Carbenoxolone was administered via the drinking water. Aspirin was suspended in 0.1N HCl and administered by stomach tube (5ml/ferret).

Homogenates of body mucosal scrapings were incubated with 1 μCi of N-acetyl-(3H)glucosamine or L-(3H)serine for 4h.

(1) p < 0.05 (Students t test)
(2) p < 0.01 (Students t test)
CONCLUSIONS

The present results on the effects of carbenoxolone sodium on gastric glycoprotein synthesis confirm those of Shillingford et al (1974) and Johnson et al (1975) who showed that carbenoxolone sodium increased the rate of incorporation of radioactively-labelled carbohydrates into glycoproteins of rat, ferret and human gastric mucosa. As it has also been shown that experimental erosions induced by stress and anti-inflammatory drugs are associated with a decrease in the rate of incorporation of labelled carbohydrate into gastric glycoproteins (Chapter 4), it is suggested that changes in the rate of incorporation of carbohydrates into gastric glycoproteins reflect alterations in the biosynthesis of mucus glycoproteins and hence in the protective capacity of mucus. This may explain the beneficial effect of carbenoxolone sodium on mucus production and its proven efficacy in the treatment of gastric ulcer.

From the above, it would be expected that cicloxolone sodium would also be clinically active in accelerating ulcer-healing as it showed a similar enhancement to carbenoxolone sodium on the rate of incorporation of N-acetylglucosamine into rat gastric mucosal glycoproteins. The results obtained with experimental ulcers and erosions support this, showing that cicloxolone has anti-ulcer activity at least equal to and possibly greater than that of carbenoxolone. Further work is in progress with cicloxolone sodium to evaluate its potential usefulness as a therapeutic agent for peptic ulcer.

The results obtained using the analogues with prostaglandin-like side chains give no indication of any stimulatory effects on the rate of glycoprotein synthesis and the inhibition shown by one of the compounds (BX 532A) would appear to be in agreement with the effects of prostaglandins shown in a previous chapter (Chapter 5).

BX 505A however, which had previously been shown by Dr. Taylor to have interesting proteolytic inhibitory activity, showed an activity of the same order as carbenoxolone and would
therefore appear to be worth further study.

The present results of the effect of carbenoxolone pretreatment on the gastric glycoprotein synthesis in the ferret confirm the findings of Johnson et al (1975) and it is particularly interesting that this increased synthesis of gastric glycoproteins is associated with a reduced susceptibility to aspirin-induced gastric mucosal damage. The response to aspirin of an increased serine incorporation without an accompanying increase in acetyl-glcuosamine incorporation is probably an attempt by the mucosa to compensate for increased secretion and effusion due to the irritant properties of aspirin. The result of this however would be synthesis of mucus deficient in carbohydrate which may make the mucosa susceptible to erosion formation. Carbenoxolone pretreatment increases the hexosamine incorporation and the newly synthesised mucus is thus more capable of protecting the mucosa against aspirin-induced damage.

Scanning electron microscopy confirmed to some extent the protective effect of carbenoxolone against aspirin-induced damage but artefacts due to the drying procedure prevented a detailed analysis. In future stereoscan studies of the gastric mucosa it is recommended that the various drying techniques be compared for their effects on mucosal structure.
7.1. Introduction

7.2. Methods for investigation of factors involved

7.3. Factors involved in the formation and healing of experimental gastric ulcers and erosions

7.4. Proposals for future research.
7. **FINAL DISCUSSION**

This chapter presents an overall view of the experimental work completed, the conclusions drawn from it and suggestions for further work.

7.1. **Introduction**

In considering the mechanisms of ulcer formation it was thought that too little attention had been paid to the role of mucus. It has long been believed that mucus is an important factor in the resistance of the gastric mucosa to peptic ulceration but there is surprisingly little firm evidence to support this view. Thus the present studies emphasize the role of mucus in the pathogenesis of peptic ulcer although it is important to realize that this is not the only factor involved. The role that the various other factors may play was considered in Chapter 1 and it is also important to realize that there is a great deal of interplay between them, as illustrated in Fig. 7.1. There is no known single causative factor of gastric ulcer but it is possible that one such factor affects several pathogenic mechanisms or leads to an ulcer when there are particular pre-existing conditions.

7.2. **Methods for investigation of factors involved**

Models involving different mechanisms for producing gastric mucosal damage were sought and high incidences of erosion or ulceration were obtained in the rat using stress, drugs (phenylbutazone, aspirin), a corticosteroid (prednisolone) and electrocautery. In a larger series of experiments it was found that the degree of mucosal damage obtained was dependent on the age, bodyweight and sex of the animals, ambient temperature and time of year. Reproducible results were obtained only by carefully controlling experimental conditions. Gross and histopathological studies of the ulcer models showed that stress, drugs and prednisolone produced superficial erosions with loss of glandular epithelium
Fig. 7.1

Interrelationships of some factors involved in the pathogenesis of gastric ulcers and erosions
but which never penetrated the muscularis mucosa. The lesions induced by electrocautery, although unphysiological, were like human chronic ulcers in appearance with destruction of the full thickness of the mucosal wall and healing taking place through processes of granulation and epithelial migration. Phenylbutazone had no effect on the healing of cautery ulcers, although it did produce superficial erosions in another part of the stomach, while cortisone exacerbated the cautery ulcers without producing any new mucosal damage. This indicates that there may be fundamental differences between superficial erosions and full thickness ulcers.

7.3. Factors involved in the formation and healing of experimental gastric ulcers and erosions

7.3.1. Corticosteroids

Corticosteroids have been implicated in peptic ulcer disease but there is apparently conflicting data on their effects on gastric mucosal integrity. The problem may be resolved if more attention is paid to the levels of corticosteroids involved and the present study gives some clues. Firstly, ulcer susceptibility is raised in the absence of endogenous corticosteroids indicating that, in physiological amounts, they contribute to the maintenance of gastric mucosal integrity. Secondly, a small excess of corticosteroids had no gross effect on normal gastric mucosa but caused increased penetration and re-exacerbation of full-thickness ulcers. Thus corticosteroids may be involved in the problems arising from chronic ulcer (delayed healing, enlargement and possibly recurrence) and avoidance of stress-induced increases in corticosteroids by bed rest is an effective measure of increasing the healing rate. Thirdly, a large excess of corticosteroids in experimental animals produced acute, non-penetrating gastric mucosal erosions and this would seem to have a clinical counterpart in stress ulcers or erosions and acute gastric mucosal bleeding.
7.3.2. Anti-inflammatory drugs

Anti-inflammatory drugs such as aspirin and phenylbutazone have long been suspected of causing acute, haemorrhagic lesions clinically but recently they have also been implicated in the pathogenesis of chronic ulcers (for example, MacDonald, 1973). It would seem that there is ample justification for further study not only for their particular pharmacological effects but also for what might be learned about ulcerogenic processes in general.

7.3.3. Mucus

Histochemically, erosion formation caused by stress, aspirin, phenylbutazone and prednisolone was associated with a loss of mucus, particularly from the regenerative zone of the mucosa. Neutral mucosubstances appeared to be affected to a greater extent than sulphated mucosubstances or sialomucins. Changes in the pattern of mucus secretion during healing of ulcers and erosions were complex and difficult to interpret but it is possible that some of the changes allowed greater secretion of mucus for protection of the regenerating epithelium.

Erosion formation was also accompanied by changes in the carbohydrate content of the mucosa indicating a decreased amount of mucus with an altered carbohydrate composition. The precise effects of an alteration in carbohydrate composition are not known but it seems reasonable to assume that they will undermine the protective capacity of the mucus. The changes were greater in rats with erosions but were also present in some extent in the rats without erosions indicating that they preceded, and were not a result of, erosion formation. These changes in mucus secretion, determined biochemically, were in good agreement with the histochemical picture. It is important to study the changes in mucus in relation to the morphology of the mucus cells as the mucus secretion is not uniform throughout the mucosa and thus misleading interpretations can be made from biochemical data alone.
7.3.4. Glycoprotein synthesis

It was decided to proceed further with the investigation of mucus by measuring its rate of biosynthesis within the mucous cells. Studies of mucus glycoprotein synthesis by measuring the rate of incorporation of a labelled carbohydrate and amino acid into gastric mucosal glycoprotein showed that erosions are accompanied by a decrease in the glycosylation of the glycoprotein whereas amino acid incorporation, i.e. polypeptide synthesis, was not reduced. The changes strongly suggest that one mode of action of stress and ulcerogenic drugs may be a decreased synthesis of glycoprotein or the synthesis of a modified glycoprotein or both. The glycoprotein synthesis could be affected by either (a) inhibition of the whole cell by a direct action on the cell wall (b) inhibition of the biosynthesis of the glycosyltransferase enzymes (c) direct inhibition of the glycosyltransferase enzymes (d) prevention of maturation of the glycoprotein-producing cells, resulting in unstable mucus being synthesised in reduced amounts. Since the synthesis of the polypeptide moiety of glycoprotein was not affected, it is most likely that only the glycosylation processes, i.e. (b) & (c) were involved.

7.3.5. Blood glucose

Blood glucose levels appear to have been little studied as a possible factor in the development of gastric lesions but the present studies showed that hypoglycaemia decreased glycoprotein synthesis and increased the susceptibility to erosion formation. Thus blood glucose levels may have an important role as it appears that a constant supply of glucose to the mucosa is necessary for synthesis of the mucous glycoprotein and normal cell function. From this it may be inferred that individuals who fail to have regular meals increase their risk factor, especially as the presence of food in the stomach itself probably also lends some protection against mucosal damage. Thus, although fasting itself for short periods does not cause gastric ulceration or haemorrhage, it may
well increase the chances of it occurring, especially in the individuals who also consume anti-inflammatory drugs, caffeine (coffee, tea), alcohol or cigarettes (nicotine).

Blood glucose levels may also be important in patients who have already developed stress ulcers as usually these patients cannot eat and require intravenous nutritional support. It is also possible that a low intake of food in stressed surgical patients may contribute to the likelihood of acute haemorrhagic gastric erosions.

7.3.6. Prostaglandins and cyclic AMP

Prostaglandins E₁, E₂, and F₂α were found to decrease the rate of incorporation of both carbohydrate and amino acid into gastric mucosal glycoproteins and may be involved in the control of glycoprotein synthesis. Prostaglandins were also found to inhibit erosion formation and this occurred at doses which also inhibited glycoprotein synthesis, suggesting that some mechanism other than stimulation of mucus production must be sought. The anti-ulcer effect of prostaglandins is commonly attributed to inhibition of acid and pepsin secretion but proof of this is lacking.

The dibutyryl analogue of cyclic AMP but not of cyclic GMP was also found to inhibit glycoprotein synthesis. It is therefore possible that the effect of prostaglandins and hypoglycaemia is mediated via stimulation of adenyl cyclase. If this were the case then both cyclic nucleotides and prostaglandins would be expected to diminish the various components of gastric secretion, but the bulk of the evidence suggests that prostaglandins inhibit and cyclic AMP stimulates gastric acid secretion. Stimulation of adenyl cyclase by the hormones released in response to lowered blood glucose levels (adrenaline, glucagon, corticosteroids) may explain the inhibition of glycoprotein synthesis by hypoglycaemia but against this is the fact that adrenaline has previously been shown to have no effect. Furthermore, administration of insulin, which might have been expected to lower cyclic AMP levels and thus stimulate glycoprotein synthesis, caused inhibition of glycoprotein synthesis.
The drug carbenoxolone sodium and an analogue, cicloxolone sodium, were found to be effective against stress-induced erosions and also to promote the healing of electrocautery ulcers. At the present time, cicloxolone is undergoing evaluation as a potential therapeutic agent with greater efficacy and less side effects than carbenoxolone. These results on erosion formation and ulcer healing suggest that cicloxolone has activity at least equal to and probably greater than that of carbenoxolone.

It is of great interest that both these drugs were shown to increase the glycosylation of mucus glycoproteins, an effect which probably explains the reported increase in mucus production after carbenoxolone treatment.

Although not the only beneficial effect of carbenoxolone on the gastric mucosa, it seems likely that the effect on glycoprotein synthesis is important for the therapeutic action of this drug. Carbenoxolone and cicloxolone may stimulate glycoprotein synthesis in several ways, namely, (a) by decreasing the extrusion rate of the epithelial cells and allowing the cells to acquire full maturity and produce a stable glycoprotein (b) a local action of the drug upon the membrane of the epithelial cells promoting cellular release of glycoproteins and thus removing possible feedback inhibition of the glycosyltransferases (c) small amounts of the drug may enter the cells and directly stimulate the glycosyltransferase enzymes.

7.4. Proposals for future research

In a recent Medical Research Council report by a specialist subcommittee on gastroenterology (Medical Research Council, 1975) it was stated that "peptic ulcer, including studies at cellular level, work on the role of mucus and experimental production and healing of ulcers", was an area meriting support for research. This has been the approach made in the present studies and further indications of directions in which to proceed have been obtained.
7.4.1. **Animal models for peptic ulcer**

In the past, the important factor of the resistance of the stomach to digestion has been greatly neglected, partly because of the lack of small animal models for peptic ulcer, since spontaneous ulcer in animals is very rare. However, although it is clear that no single ulcer models meets all requirements, investigations of the defensive factors in the existing small animal models for erosions and ulceration may prove of fundamental importance to the peptic ulcer problem. At the same time development of new models more akin to human peptic ulcer would no doubt lead to a great advance in understanding of the disease.

7.4.2. **Mucus**

The present methods of the characterisation of the gastric mucus are incomplete and a more detailed characterisation can be obtained with gas-liquid chromatography. It would seem that detailed knowledge of the chemical composition and structure of mucus is necessary for a full understanding of its function.

The method used in the present studies for measurement of the synthesis of glycoproteins should be followed up and improved. A more selective precipitation and fractionation of the gastric mucosal glycoprotein is required. The method could also be modified to measure synthesis and secretion of the glycoproteins in the same tissue samples. The synthesis of glycoproteins in human gastric biopsies is being studied by this method and this would seem to be an important approach and one which should be pursued. Glycosyltransferases and other enzymes involved in the synthesis of structural and secreted glycoproteins should also be studied, initially in animals and eventually in human gastric biopsies.

The present work showed that stress and drugs inhibited glycoprotein synthesis and the intriguing question arises as to whether not only mucus synthesis but also cell turnover, regulated by surface membrane glycoproteins, is affected by this mechanism.
The role of the cell membrane glycoproteins may turn out to be more important than that of the secreted ones.

It must be borne in mind that in future studies biochemical studies of mucus should be correlated with morphological changes.

7.4.3. Blood glucose levels

Many studies on the effect of nutrition in peptic ulcer have failed to separate the effects of carbohydrates, proteins and vitamins. It appears from the present studies that blood glucose levels may be an important factor and it would be desirable to confirm this work and investigate the clinical implications.

7.4.4. Prostaglandins and cyclic nucleotides

The present studies cast doubt on any physiological role of prostaglandins or cyclic nucleotides in mucus synthesis and secretion. However, bearing in mind the studies performed to determine the roles of prostaglandins and cyclic nucleotides in acid secretion, and the inconclusive and contradictory results from these studies, it is likely that much more work requires to be done before conclusive evidence is obtained.

7.4.5. Carbenoxolone

The protection by carbenoxolone against aspirin-induced erosions in the ferret confirms the suitability of the animal in gastroenterological research. It appears to respond better to the effect of carbenoxolone than does the rat and so more closely resembles man in this respect. It is therefore recommended that further studies be carried out using the ferret to determine whether this species should replace the rat, cat or dog in gastroenterological research.

From the results of these and other studies on glycoprotein synthesis, it is possible that there is a further use for
Carbenoxolone as follows. Carcinogens are capable of damaging the endoplasmic reticulum. This effect may be related, if not directly equated, to the process of "degranulation", that is, the removal of the ribosomes from the endoplasmic reticulum. This process, which converts the "rough" endoplasmic reticulum to the "smooth" is associated with a switchover of cellular protein synthesis away from proteins secreted by the cell e.g. glycoproteins, to proteins required for the cell's own structure and function.

The process of removal of the ribosomes from the membrane is reversible and is regulated by steroid hormones although the removal by carcinogens is thought to be irreversible. As carbenoxolone has been shown to have a specific action in increasing the synthesis of glycoproteins secreted by the gastric mucosal epithelium, and has been shown to potentiate corticosteroid activity, it may have the property of regulating the ribosomes on the endoplasmic reticulum and may thus influence the effects of carcinogens.

Studies of the effects of carbenoxolone on carcinogenesis are awaited with interest.
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