DRUG EFFECTS ON THE GASTRIC MUCOSA
IN RELATION TO ULCEROGENESIS

by

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A thesis submitted in accordance with the requirements of the University of Surrey for the degree of Doctor of Philosophy.

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Guildford, Surrey. January, 1977
SUMMARY

It has been suggested that a gastric ulcer results when the ability of the stomach to defend itself against the aggressive forces of the gastric contents breaks down. The studies in this dissertation are concerned with the effects of agents on the synthesis of mucosal glycoproteins, the principle components of gastric mucus which constitutes the first line of defence of the gastric mucosa.

Studies on the synthesis of mucosal glycoproteins, employing the in vitro incorporation of radiolabelled precursors, showed that human gastric ulcer was associated with a derangement of mucus synthesis characterised by decreased incorporation rates of N-acetylglucosamine. In contrast sulphate uptake appeared to be increased in disease states.

Whereas predosing animals with mucosal damaging agents, such as ethanol, or a period of starvation, produced reductions, carbenoxolone and the E type prostaglandins, which have been shown to be beneficial in the treatment of ulceration, caused marked increases in the rate of incorporation of N-acetylglucosamine.

Further studies with carbenoxolone indicated that the effect of the drug is on the degree of glycosylation of the glycoprotein rather than on the amount produced. It is suggested that carbenoxolone results in the production of a modified glycoprotein carbohydrate side chain, richer in N-acetylglucosamine, N-acetylgalactosamine, N-acetyleneuraminic acid and fucose and depleted with respect to galactose. The drug also resulted in increased sulphation of mucosal glycoprotein.
Carbenoxolone has also been investigated in relation to its effect on the release of lysosomal enzymes which have been shown to be elevated in drug induced gastric mucosal lesions. The results show that the drug is extremely membrane active causing lysis of lysosomal membranes at high concentrations (above $10^{-4} \text{M}$) and stabilization at lower concentrations ($10^{-5} \text{M}$).

The significance of these findings with relation to ulceration and the beneficial action of anti-ulcerogenic drugs is discussed.
TO MY PARENTS
Acknowledgements

I would like to thank Professor D.V. Parke for his encouragement and advice and for giving me the opportunity to work in his Department.

I am grateful to Dr. A.M. Symons for his helpful supervision and encouragement and to Dr. W.E. Lindup for introducing me to the field of gastric mucus. I would also like to thank Dr. M. Smith of St. Luke's Hospital, Guildford, for the provision of human gastric biopsy material and Mr. P. Scobie-Trumper and his staff of the University of Surrey Animal Unit.

I am particularly grateful to Mrs. M. Whatley both for her many words of wisdom throughout my time at the University of Surrey and for typing this thesis.

Finally, I am indebted to Biorex Laboratories Limited for the financial support they have provided.
Abbreviations and Conventions

In general abbreviations comply with those suggested by the Biochemical Journal.

This thesis is divided into five chapters. The bibliography follows Chapter 5. Tables and figures have been numbered 1.1 etc., the first number referring to the chapter and the second to the order within the chapter.

Publications

Some of the work in this dissertation has been published in the following:-


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CHAPTER ONE
GENERAL INTRODUCTION

1.1 The Stomach - structure and function
1.2 Ulceration
1.3 Carbenoxlone Sodium
1.4 Aim of project
THE STOMACH - STRUCTURE AND FUNCTION

The stomach develops as an unequal dilatation of the foregut. The ventral and dorsal surfaces may be vaulted or flattened and may touch when the organ is empty. It has two borders; the concave lesser curve and the convex greater curve. These join at the cardia where the oesophagus enters (Fig. 1.1). On the right the oesophagus continues smoothly into the lesser curvature but on the left there is a definite indentation known as the cardiac incisure.

The major portion of the stomach, known as the corpus or body, blends imperceptibly with the pyloric portion, except along the lesser curvature, where the angular incisure marks the boundary between the corpus and the pyloric region. The latter is divided into the pyloric antrum or vestible which narrows into the pyloric canal, terminating at the pylorus.

The mammalian gastric mucosa may be classified as glandular or non-glandular. The former is present in all stomachs and is responsible for the secretion of gastric juice. Non-glandular mucosa is found in certain species only (e.g. the fundic region of the rat, mouse and guinea pig stomachs).

The mucous membrane of the stomach, composed of a single layer of epithelial cells, the tunica propria and the submucosa, commences at the cardia along an irregular zig-zag line (the z line). Here the mucosa consists of marked reliefs of rugae, which flatten considerably as the stomach is distended. In the region of the lesser curve where the mucosa is strongly fixed to the muscular layer, the folds take a longitudinal course, forming the "Magenstrasse". The rugae are smaller in the fundus and become larger as they approach the antrum where they tend to run diagonally across the stomach towards the greater curvature.
Figure 1.1  THE HUMAN STOMACH

- Fundus (Fornix)
- Cardia (cardiac orifice)
- Cardiac incisure
- Body (corpus)
- Angular incisure
- Pyloric canal
- Pyloric portion
- Pyloric antrum
- Lesser curvature
- Greater curvature
- Duodenum
Besides these broad folds the mucosal surface is further characterized by numerous shallow invaginations. These areae gastricae contain delicate ledges and depressions, the latter known as the gastric pits or foveolae gastricae. In the depths of these pits the glands of the stomach open.

At the gastro-oesophageal junction the gastric epithelium, consisting of a single layer of columnar cells, is sharply demarcated from the stratified and thicker oesophageal mucosa. The epithelial cells are of the mucoid type, containing mucinogen granules in their outer portions and a single ovoid nucleus at their base.

The glands of the stomach are tubular and can be sub-divided into three types (Fig. 1.2) -

(1) The cardiac glands. These are confined to a narrow zone, 0.5 to 4 cm in width, around the cardiac orifice. They are coiled and lined by mucus producing cells.

(2) The gastric or fundic glands. These are located in the fundus and cover the greater part of the body of the stomach. They are reasonably straight, simple branched tubules, with a narrow lumen reaching down almost to the muscularis mucosa. Three types of cell are found:–

(a) Mucus secreting cells. The neck of the gland is lined by mucus secreting cells. These differ from the cells of the surface epithelium in that their mucinogen granules have slightly different staining properties and their nuclei tend to be flattened or concave rather than oval.

(b) Chief cells. The chief or zymogenic cells line the lower half of the glandular tubules. They have spherical nuclei, contain dense
Figure 1.2  Glands and Cell Types in the Gastric Mucosa

- Cardiac Glands
- Pyloric Glands
- Surface Epithelial Cell
- Mucous Cell
- Parietal Cell (between a mucous cell and a zymogen cell)
- Zymogen Cell
- Solitary Lymph Nodule
- Argentaffine Cell
- Muscularis Mucosae
- Submucosa
- Gastric or Fundic Glands
light-refracting granules and golgi apparatus, the size and form of which vary with the state of secretory activity. These cells are responsible for pepsinogen secretion.

(c) Parietal cells. These cells are longer than the chief and mucus secreting cells and are usually crowded away from the lumen to which they connect by a series of extracellular capillaries, stemming from intracellular canaliculi. The parietal, or oxyntic cells are responsible for the production of gastric hydrochloric acid.

and (3) The pyloric glands. These are located in the pyloric region but also spread into a transitional zone, in which both gastric and pyloric glands are found and which extends diagonally and distally from the lesser to the greater curvature. The tubes of the pyloric glands are shorter, more tortuous, less densely packed and less branched than the fundic glands. These glands are lined by a single type of cell, which is mucus secreting and resembles closely the mucous neck cells of the fundic glands.

Three other cell types are present in the human antrum; i) argentaffine cells, which contain 5-hydroxy-tryptamine and are found in the deeper glands; ii) apud cells, which are morphologically related to the argentaffine cells and may contain histamine, and (iii) cells provisionally termed 'S' cells, which are found at the base of the gastric pits. 'S' cells are thought, mainly on the basis of the vesicles and fibres they contain, to be either secretory cells, monitoring the acidity of the gastric mucosa, or cells producing secretin.

The secretory products of all these various cells constitute gastric juice. The cell types and their secretory products are summarized in Table 1.1.
Table 1.1 Cell types found in the gastric mucosa and their secretory products

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Product(s)</th>
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<tr>
<td>Surface mucous</td>
<td>Mucus</td>
</tr>
<tr>
<td>Mucous</td>
<td>Groups I and II pepsinogens, mucus</td>
</tr>
<tr>
<td>Oxyntic (parietal)</td>
<td>Acid, intrinsic factor</td>
</tr>
<tr>
<td>Chief (zymogen)</td>
<td>Groups I and II pepsinogens</td>
</tr>
<tr>
<td>Argentaffine</td>
<td>Serotonin, histamine</td>
</tr>
<tr>
<td>Gastrin</td>
<td>Gastrin</td>
</tr>
<tr>
<td>Pyloric gland (mucous)</td>
<td>Group II pepsinogen, mucus</td>
</tr>
</tbody>
</table>
Gastric secretion is under both nervous and hormonal control. The neural control is via the vagus nerves. Vagal activity or distension of the stomach wall by food stimulates exocrine secretion of acid and pepsin, endocrine secretion of gastrin, and sensitizes parietal cells to the action of gastrin.

When stimulation fades and inhibition occurs, gastric secretion subsides. Satiety stops cortical stimulation of the vagi and the low pH of the gastric contents prevents further release of gastrin. Hormonal inhibitors of secretion are secretin and cholecystokinin. The duodenal mucosa releases these hormones when food and gastric juice pass from the stomach.

There is some evidence to suggest that a second messenger hypothesis for mediation of cellular responses to hormones and other agents may apply to oxyntic cells. It has been suggested that cyclic AMP fulfills this role (Harris et al., 1965; 1969) via its action on carbonic anhydrase (Bersimbaev et al., 1971; Salganik et al., 1972). This enzyme plays an important role in the secretion of the HCl, being involved in the formation of hydrogen ions and their transport through the cell wall.

Cyclic AMP mediates the action of a number of hormones by activation of protein kinases in target cells. The protein kinases in turn phosphorylate definite enzymes or other proteins, resulting in the physiological effect of the hormone. It is possible that changes in carbonic anhydrase activity produced by cyclic AMP may also arise as a result of such an enzymic protein phosphorylation.
Bersimbaev and his co-workers (1971) have proposed a scheme to explain the effects of various secretagogues on cyclic AMP and gastric acid production in the rat. In this scheme gastrin and histamine stimulate the production of cyclic AMP, the second messenger, which controls the production of HCl by its effect on carbonic anhydrase. The hypothesis is supported by their finding that theophylline, an inhibitor of phosphodiesterase augmented the effect of the secretagogues on acid production (see Fig. 1.3).

This second messenger role for cyclic AMP has received support from a large number of authors, with regard to acid secretion by the rat (Domschke et al., 1973; Puurrenen and Karppanen, 1975), guinea pig (Karppanen et al., 1974), rabbit (Fromm et al., 1975) and frog gastric mucosae (Harris et al., 1965; 1969). Conflicting findings, however, have been reported for canine (Levine and Wilson, 1971; Mao et al., 1973) and human gastric mucosae (Domschke et al., 1974; 1975). The evidence for and against the role of cyclic AMP in the control of acid secretion has been reviewed recently (Kimberg, 1974).

Recently, a large volume of work has been devoted to elucidating the role of prostaglandins in the stomach. Of the prostaglandins which can be isolated from the gastric mucosa of the rat (PGE$_1$, PGE$_2$, PGF$_{1a}$ and PGF$_{2a}$, Shaw and Ramwell, 1968), PGE$_1$ and PGE$_2$ have been shown to inhibit secretion in rats (Robert, 1968; Shaw and Ramwell, 1968; Main and Whittle, 1973), dogs (Robert et al., 1967; Nezamis et al., 1971) and humans (Claussen et al., 1970).

Another naturally occurring series of prostaglandins, PGA$_1$ and PGA$_2$, which have also been studied with regard to gastric secretion, causes inhibition in rats (Main and Whittle, 1973), dogs (Robert et al., 1967) and humans (Wilson et al., 1971).
Fig. 1.3  Scheme for the regulation of HCl secretion

Gastrin (gastrin pentapeptide)

↓

Histidine decarboxylase

Histidine → Histamine

ADP → Cyclic 3'5'-AMP

Carbonic Anhydrase → HCl

Phosphodiesterase → 5'-AMP

Methylxanthines (theophylline, caffeine)
The available evidence suggests that the prostaglandins possess a broad spectrum of anti-secretory activity rather than a specific antagonist action. Whereas PGE$_1$ effectively inhibited gastric secretion induced by a number of secretagogues including histamines and food, in the dog, PGA$_1$ was effective against food-induced but not histamine-induced secretion (Robert et al., 1967). Furthermore, in human studies PGA$_1$ has been shown to inhibit histamine-induced secretions (Wilson et al., 1971).

The mechanism of the anti-secretory action of prostaglandins is not known. They have potent effects on the gastric mucosal blood flow in dogs (Robert et al., 1967) and the rat (Main and Whittle, 1972, 1973a, 1973b) but this effect may be to increase or decrease the blood flow, depending on the dose of prostaglandin and is believed by these authors to be secondary to the inhibition of acid secretion.

Evidence has accumulated suggesting that the prostaglandins act through cyclic AMP. Thus the infusion of prostaglandin E$_2$ into dogs with Heidenhain pouches significantly decreased the amount of cyclic AMP secreted into the gastric juice as well as the amount of acid secreted (Bieck et al., 1973). Other experimental studies, however, have failed to demonstrate the importance of cyclic AMP in the control of canine gastric secretion (Mao et al., 1972).

In the rat cyclic AMP induced secretion is partially inhibited by PGE$_2$ (Whittle, 1972) and dibuteryl cyclic AMP stimulated secretion can be antagonised by PGE$_1$ (Jawaharlal and Berti, 1972). In contrast PGE$_1$ inhibited gastrin and histamine but not cyclic AMP induced-secretions in the frog stomach (Way and Durbin, 1969).
Thus the interaction of prostaglandins with cyclic AMP levels may be different in different species and the evidence in favour of such an interaction playing an important role in the control of acid secretion remains unconvincing.

**Gastric Mucus**

Gastric mucus is secreted by the cells of the surface epithelium and those lining the crypts of the mucosa and pyloric glands. It is highly alkaline and viscous, covering the surface of the mucosa with a tenacious layer about 1 mm thick.

It is firmly established that glycoproteins are the principle components of gastric mucus (Spiro, 1970). Studies on mucosubstances from the stomach of the pig, which physiologically is closely related to that of man (Montagna, 1959) indicate that gastric mucus contains two glycoproteins of molecular weight $2.3 \times 10^6$ and $1.1 \times 10^5$ (Allen and Snary, 1972). These mucoproteins are closely related, being identical in A and H blood group activities, chemical analysis and in many physical properties (Snary and Allen, 1971). For these reasons the high molecular weight glycoprotein is thought to be a polymer of the low molecular weight molecule.

These glycoproteins are characterised by a high content of serine, threonine and proline, which together account for 40% of the total amino acids present (Starky et al, 1972). Serine and threonine are thought to be the amino acids involved in the peptide-carbohydrate link (Marshall, 1972; Slomiany and Meyer, 1972). The sum of these two amino acids approximately equals the number of N-acetyl-D-galactosamine residues, a relationship which is thought to indicate the involvement of this residue in the linking of the side chains (Schrager, 1969).
carbohydrate moieties involved in the side chains of gastric mucosal
glycoproteins are galactose, fucose, glucosamine, galactosamine
and sialic acid (Schrager and Oates, 1970).

The protection afforded by the mucus layer is partly mechanical,
preventing access of acid and pepsin to the epithelial cells (Johnson,
1957) and partly chemical because it can neutralise acid to some extent;
the former function, however, is more important than the latter (Grossman,
1958).

That the carbohydrate side chain plays an important role in the
protection afforded by mucus is evidenced by the work of Gottschalk
(1960) who found that the viscosity of mucus, an important factor in
its adhesion to the mucosa, is dependent on the sialic acid content of
the glycoprotein. The role of sialic acid in maintaining the integrity
of the mucus coat was further supported by Domschke et al (1972) who
found that N-acetylneuraminic acid levels were significantly decreased
in the mucus of gastric ulcer patients.

Studies with ulcerogenic drugs have also demonstrated the protective
function of mucus and the importance of the carbohydrate side chains in
maintaining this function. Aspirin (Menguy et al, 1965), phenylbutazone
(Menguy and Desbaillets, 1967a) and indomethacin (Menguy and Desbaillets,
1967b), which lead to gastric lesions, cause a decrease in the synthesis
of mucus and also a decrease in the concentration of protein bound
carbohydrates, particularly sialic acid and hexosamines. Moreover, the
same authors (Menguy and Desbaillets, 1968) found that mucus which was
deficient in carbohydrate was more susceptible to proteolytic digestion.

The structure, function and biosynthesis of glycoproteins and the
effects of agents upon these aspects of gastric mucus will be discussed
in fuller detail in Chapter 2.
1.2 ULCERATION

Peptic Ulcer. Peptic ulcer can be defined as a sharply circumscribed loss of tissue, involving the mucosa, submucosa and muscular layer, occurring in areas of the digestive tract exposed to acid and pepsin containing gastric juice. Thus the disease may occur in the lower part of the oesophagus, the small intestine and the stomach. In the latter region the lesion constitutes a gastric ulcer.

Pathophysiology of the gastric ulcer

Most gastric ulcers occur along the lesser curvature of the stomach and are associated with hyposecretion of acid. Johnson (1965) found that this group comprised 57% of his large series of 5,000 cases.

Acute and chronic ulcers differ chiefly in the amount of granulation tissue and fibroplasia. Acute erosions extend only to the muscularis mucosa and superficial ulcers to the upper portion of the submucosa. They are usually multiple, oval or round and vary in diameter from several millimeters to one or two centimeters.

Chronic gastric ulcer is usually single, but may be multiple. The crater is oval or elliptical, with overhanging edges. Crater diameter may vary from several millimeters to several centimeters and the depth from 10 to 20 mm or more. The adjacent mucosa may be normal, inflamed or atrophic. Microscopically, chronic gastric ulcer is a "U" shaped defect, extending through the mucosa and submucosa to a varying depth in the muscularis propria. Occasionally it may penetrate the serosal layer, invading the pancreas or liver.

The ulcer base typically comprises four zones: on the surface a greyish exudate of leukocytes, overlying a zone of fibrinoid necrosis,
superimposed on a layer of granulation tissue which blends with the underlying cicatrical zone. The muscular layers are interrupted completely by the ulceration and fibrous tissue. Activity and healing co-exist in the same lesion.

In acute ulcers the blood vessels are normal but in the subacute and chronic lesions the vascular changes may include partially organised thrombi, arteritis, periarteritis and subintimal proliferation of connective tissue. There may also be inflammation and degeneration of nerve fibres and disappearance of ganglion cells.

**Distribution**

Knowledge of the gastric ulcer apparently dates from antiquity, but the reports were vague until the report of Mathew Baillie (1793). Cruveilhier in 1829 described the lesion in detail and Brinten published a comprehensive account in 1857, based on more than 7,000 autopsies.

Peptic ulceration is a disorder of man. Erosions and superficial lesions occur spontaneously in many species but chronic ulcers are rare. The disease is a common one, the incidence being possibly as high as 10% (Doll et al., 1951) of the population. Before 1900 the frequency of gastric ulcer exceeded that of duodenal ulcer and females were affected more often than males. These trends have now been reversed - ratio of duodenal ulcer to gastric ulcer 10:1 (Watkinson, 1960) - except in Japan where gastric ulcer is more common than duodenal ulcer (Rhodes, 1972) and in Finland where the incidence is approximately equal. Gastric ulceration is also very common among Indians of the Peruvian Andes, developing at an early age and often being complicated by haemorrhage. The high altitude, the coarse nutritionally inadequate diet, chewing of cocoa beans, hot alcoholic beverages and hazardous work have all been implicated as causative factors.
Peptic ulcers occur at all ages but symptoms usually develop between the ages of 20 and 40. The highest incidence for the disease occurs between the ages of 45 and 55. Until puberty, the sex distribution of peptic ulcer is the same. After puberty the incidence of the disease increases in both sexes, but more in males than females. Among adults, males predominate in a ratio of 4:1 for gastric ulcer. After artificial or spontaneous menopause the incidence of ulceration in women rises. Active ulceration is a rare phenomenon during pregnancy but may recur in the last trimester or early puerperium. These observations in women indicate a sex-linked protection against ulceration.

The striking incidence of peptic ulcer in some families, the frequency among living siblings of ulcer patients and the occasional ulcers in homozygous twins indicate genetic influences. There is an interesting relationship between the distribution of blood groups in patients with peptic ulcer. Individuals who belong to blood group 0 and whose genes determine that they do not secrete A, B or H blood group substances, have a 2.5 times greater risk of developing ulcers than persons of blood groups A or AB who are secretors of these substances.

The Gastric Mucosal Barrier

It has been stated that peptic ulceration is a common lesion. One of the most interesting facets of the disease is why it does not occur more frequently than it does, in view of the digestive capabilities of the gastric contents. Between the stomach contents and the plasma there is a $H^+$ gradient of over 1 million to 1. The remarkable ability of the stomach to maintain this gradient is due to the gastric mucosal barrier, a concept at first proposed by Teorell (1933). This barrier is dependent on two factors, the superficial layer of mucus which covers the epithelium
Table 1.2 The Aggressive and Defensive Factors Associated with Gastric Ulceration

<table>
<thead>
<tr>
<th>Aggressive Factors</th>
<th>Defensive Factors</th>
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<tbody>
<tr>
<td>Increased acid production</td>
<td>Decreased acid synthesis</td>
</tr>
<tr>
<td>Increased pepsinogen synthesis</td>
<td>Decreased pepsin synthesis</td>
</tr>
<tr>
<td>Decreased mucus synthesis</td>
<td>Increased mucus synthesis</td>
</tr>
<tr>
<td>Refluxed duodenal contents orally ingested chemical irritants (e.g. aspirin)</td>
<td>Increased stability of mucosal cells</td>
</tr>
</tbody>
</table>

(From Bralow, 1969)
(see section 1.1) and the remarkable regenerative ability of the gastric mucosal epithelium (Lipkin, 1971).

The cellular defence mechanism depends on the very rapid turnover of cells in the gastric mucosa. Lipkin and Bell (1968) have demonstrated the dynamic and rapid life-cycle of the mucus producing gastric epithelial cells in man and other species. New cells are produced in the gastric pits and rapidly move to the surface to replace those extruded into the lumen. As the new cells migrate they undergo differentiation, which enables them to produce mucus. At the same time there is a decrease in DNA synthesis (Lipkin, 1971). These cells have a short life span, being extruded into the lumen within 3 days, but they are constantly being replaced by new migrating cells.

Mechanisms of Gastric Ulcer Formation

An ulcer develops in a stomach, when at a circumscribed site the resistance of the mucosa to acid and pepsin breaks down. Thus a prerequisite for the development of an ulcer is the digestive juice of the stomach and "no acid - no ulcer" (Schwartz, 1910) is the only unchallenged basic principle of ulcer formation.

The disease appears to be dependent on the interplay of a number of factors, nutritional, vascular, nervous, humoral and chemical and results from an inability of the stomach to cope with the many aggressive factors with which it comes into contact during normal function. Whether the ulcer results from an increase in the aggressive forces or a decrease in the defensive capabilities of the stomach is not clear. A summary of these factors is shown in Table 1.2.

Hyperacidity - The theory of Cruveilhier (1840)

According to this theory gastric ulcers are due to an abnormally high level of acid secretion by the stomach which overwhelms the mucosal defence.
However, gastric ulcer patients have acid secretion rates within the normal range (Wormsley and Grossman, 1968) and antacid therapy has been ineffective in accelerating the rate of healing of the gastric ulcer (Baume and Hunt, 1969). These findings have led a number of authors to conclude that hypersecretion and hyperacidity are not, in general, the cause of gastric ulceration.

The observations of Wormsley and Grossman (1968) however, refer to gastric ulcer as shown by occidental subjects. A different situation, in which gastric ulcer is associated with above normal acid secretion rates, is found in the Chinese (Fung, 1970). Similarly, the finding that gastric ulcers may be associated with extreme hypersecretion (greater than 10 mEq/hour) as in the Zollinger-Ellison syndrome (Ellison and Wilson, 1964) and that achlorhydria induced by radiotherapy caused temporary healing of gastric ulcers (Rickets et al, 1949) indicate the importance of gastric acid in the development of an ulcer.

**Antral Stasis. Dragstedt Theory**

According to this theory the following chain of events occurs: hindrance of gastric emptying - antral stasis - distension - secretion of gastrin in the antrum - hypersecretion - gastric ulcer (Dragstedt, 1956). More recent observations suggest that this mechanism, although theoretically possible, rarely occurs in practice. Delayed emptying and stasis may arise as complications of ulcers located near the pylorus but, in such cases, have nothing to do with the pathogenesis of the ulcer (Mangold, 1958). Hypergastrinaemia is frequently observed in gastric ulcer, but is the result of hyposcretion with a high pH in the antrum (James, 1949) and not the cause of hypersecretion (Trudeau, 1971).
Back Diffusion of Hydrogen Ions

In 1933 Teorell found that the gastric mucosa of cats was permeable to hydrogen ions which were exchanged for intracellular sodium ions. He postulated that the ions were exchanged by passive diffusion of hydrogen ions. Davenport (1964) demonstrated that damage to the mucosa in Heidenhain pouches in dogs caused by acetyl salicylic acid or bile salts caused the hydrogen ion efflux from the lumen to increase. He showed that a significant level of back diffusion occurred following installation of an acid solution into the stomachs of gastric ulcer, gastritic and hypochlorhydric patients. Similar results were obtained when the gastric mucosa was damaged by salicylate treatment (Ivey, 1972) and bile salts (Den Besten, 1972). It has been postulated that an abnormally permeable mucosa may exist or can be induced in humans, a phenomenon which may explain the apparent achlorhydria frequently encountered in gastric ulcer patients (Davenport, 1965; Ivey, 1971).

From these observations it may be concluded that the aetiology of the gastric ulcer in some patients could be due to back diffusion of hydrogen ions through a defective mucosal barrier.

Reflux of Bile - Du Plessis Theory

According to Du Plessis (1965) the following chain of events occur: pyloric incompetence - reflux of bile - damage to mucosal membrane - chronic gastritis spreading upwards from the pylorus - development of an ulcer in the damaged mucosa.

The evidence in favour of this theory is based on the finding that bile reflux is present in the majority of gastric ulcer patients (Du Plessis, 1965) but is rare in normal subjects.
This theory found support from the work of Davenport (1968) and Geall (1970) who discovered that bile damaged mucosa became freely permeable to the movement of hydrogen ions. In the case of massive exposure to bile, e.g. after gastric resection, intracellular mucus disappears from superficial cells (van Geertruyden, 1961) and an accelerated desquamation occurs (Castrup, 1974). It is also worth noting that ulcers can be produced more easily in mucosa previously damaged by bile than in healthy mucosa (Du Plessis, 1965; Stadelmann, 1971).

There is, however, no proof that an incompetent pyloric sphincter is the cause of gastric ulceration, rather than the result. Similarly, mucosal damage by bile has only been demonstrated in acute experiments. A causal relationship between the obligatory chronic gastritis in ulcer and the effect of bile is possible (van Geertruyden, 1961) but as yet unproven. It is equally possible that the observed gastritis is a consequence of the ulcer rather than the cause.

In addition to bile damage of the mucosa it has been suggested that duodenal reflux plays an important role in the pathogenesis of gastric ulcer due to the pancreatic secretions it contains (Capper, 1967). This hypothesis postulates that pancreatic secretion exposes the ulcer site to proteases which are active at neutral and higher pHs, resulting in a continuation of autodigestion at pH levels where peptic digestion is impossible.

**Stress Ulceration**

It is well known that severe physical stress (injuries, burns or major operations) particularly when associated with blood loss may be followed by the development of acute haemorrhagic gastric mucosal lesions (Harjola, 1966; Goodman, 1968).
The pathogenesis of the lesion is not clear but differs from that of the chronic gastric ulcer in that the primary aetiological factor appears to be a decreased mucosal blood flow (Chui, 1971), presumably due to splanchnic vasoconstriction and diversion of blood from the mucosa through arteriovenous shunts. Menguy and Masters (1974) have suggested that the link between gastric mucosal ischaemia and the eventual lesion is a severe disturbance in the energy metabolism of the gastric mucosal cell.

**Treatment of Gastric Ulcer**

There are three aims in the management of peptic ulcer, namely, the alleviation of symptoms, the healing of the ulcer and the prevention of its recurrence. While there are effective methods for relieving symptoms and accelerating ulcer healing there are no means of preventing ulcer recurrence, nor of altering the natural tendency to remit and relapse.

Methods used to alleviate symptoms and accelerate ulcer healing include bed rest, alterations to diet, cessation of smoking, major surgery and drug therapy. Bed rest is undoubtedly the single most effective measure for the relief of pain and promotion of healing. It has been shown that reflux of duodenal contents into the stomach is markedly lowered in the recumbent position (Capper, 1967; Flint and G ee ch, 1970). There is also good evidence that stopping smoking accelerates the rate of healing of gastric ulcers (Doll et al, 1958).

**Surgical Treatment**

Modern surgical treatment has much to offer the patient with intractable peptic ulceration. For gastric ulcer the operation of choice is a partial gastrectomy, preferably with a Billroth I anastomosis,
in which the ulcer itself and the ulcer bearing area of the stomach is resected. However, major surgery does have certain disadvantages in that it is irreversible and is prone to complications such as perforation, anaemia and nutritional impairment. Drug therapy, on the other hand, provides relief and promotes healing with the advantage of minimal, if any, hospitalization and subsequent major surgery.

Drug Therapy

The dominance of conventional alkali and anticholinergic therapy in the management of gastric ulceration has been challenged in the last decade by a wide range of newly developed drugs. The list is formidable and includes antipepsins and antigastrins, drugs enhancing mucosal resistance and ulcer insulation. In addition preparations are available which act on corticohypothalamic pathways and gastric motility. A comprehensive review of gastric anti-secretory and anti-ulcer agents is available (Bass, 1974).

Of particular interest and importance are the group of compounds which have a protective effect on the gastric mucosal barrier, thus enhancing its resistance to attack and accelerating the rate of healing of the gastric ulcer. This group includes, carbenoxolone sodium (Biogastrone), proglumide (Milid, Xylamide) geranyl farnesylacetate (Gefarnate, Gefarnil), cholestyramine (Questran) and possibly the E type prostaglandins (Fig. 1.4). The drug carbenoxolone sodium is the major drug of the group and has been recognised as the most striking therapeutic advance in the treatment of the disease.
Figure 1.4  Some Drugs used in the Treatment of Gastric Ulcers

(1) Carbenoxolone sodium

Proglumide

(N'-Benzoyl-N',N'-dipropyl-DL isoglutamine)

Geranyl Farnesylacetate (Gefarnate)

Cholestyramine

Prostaglandin E₁

Prostaglandin E₂
Carbenoxolone (3-O-[B-carboxypropionyl]-11-oxo-18β-olean-12-en-30-oic acid) is the synthetic hemisuccinate of the triterpenoid 18β-glycyrrhetic acid (enoxolone) which is the aglycone of glycyrrhizic acid, a substance present in licorice root. It is thus a pure synthetic derivative of a naturally occurring plant material.

To date there are more than 350 publications on the biochemistry, pharmacology and therapeutic evaluations of this product. These have been discussed at four symposia (Robson and Sullivan, 1968; Baron and Sullivan, 1970; Avery-Jones and Sullivan, 1972 and Avery-Jones and Parke, 1974). A number of review articles are also available (Sircus, 1972; Lewis, 1974; Pinder et al, 1976).

Pharmacology and Biochemistry

Carbenoxolone has anti-inflammatory properties having about one third of the activity of cortisol when administered parenterally (Khan, 1968; Sullivan, 1972). This action is thought to be partially dependent on endogenous corticosteroid production. The effects of carbenoxolone on corticosteroid levels are not apparent with acute administration (Mollman, 1973), but become obvious after prolonged treatment for a month or more (Gollan et al, 1975).

The drug also exhibits an anti-diuretic action with sodium retention and potassium depletion. This, the most common adverse reaction in carbenoxolone therapy, is manifest only at high dose levels and can be kept to a minimum by dietary management of salt intake (Banks and Marks, 1970).

Carbenoxolone is a large highly lipophilic molecule but is nonetheless extensively and rapidly absorbed in man after oral administration. An
average level of 15 µg/ml, equivalent to 75% of the dose present in blood, is reached within two hours of a 100 mg dose (Baron et al., 1975). The principal site of absorption is thought to be the stomach.

Due to the high degree of protein binding of carbenoxolone to plasma proteins, more than 99.95% bound in the dog, rabbit, rat, rhesus monkey and man (Parke and Lindup, 1973), the drug is confined almost entirely to the gastrointestinal tract, the liver and the plasma. In fact it has been suggested that there may be protein binding sites in the stomach which have a special affinity for carbenoxolone since [¹⁴C]carbenoxolone administered intraperitoneally migrates back into the gastrointestinal lumen, especially the gastric mucosa (Parke, 1972).

In the rat carbenoxolone is extensively metabolised to enoxolone and succinate (60-75% of an oral dose) by microfloral hydrolysis and is excreted in the bile, mostly as sulphate and glucuronide conjugates of enoxolone (Iveson et al., 1971). In contrast, man, the squirrel monkey and ferrets metabolise carbenoxolone primarily by conjugation to give carbenoxolone-30-glucuronide. Hydrolysis of the ester to enoxolone and succinate occurs only to a small extent, 10-20% of the dose (Iveson et al., 1971, Parke, 1972). In vitro experiments have shown that rat faeces, caecal contents or gastric aspirates readily hydrolysed carbenoxolone to glycyrrhetinic acid and succinate, whereas human blood, rat blood and rat liver were incapable of carrying out this reaction (Iveson et al., 1971).

Carbenoxolone in the Treatment of Gastric Ulcer

The first controlled trial of carbenoxolone in the treatment of gastric ulcer was reported by Doll and his co-workers (1962). Since then trials have been conducted in a large number of patients. Reviews by Piper and Heap (1972) and Pinder et al. (1976) tabulate and discuss the
clinical trials carried out on carbenoxolone. They provide good evidence for the effectiveness of carbenoxolone in accelerating the rate of gastric ulcer healing in the ambulatory patient.

Possible Modes of Action

The precise mode of action of carbenoxolone is unknown. If, however, the view is accepted that an ulcer results from an upset in the balance between aggressive and defensive forces, a number of possibilities become apparent.

Gastric Acid Secretion

Carbenoxolone has equivocal effects on the secretion of gastric acid and it seems unlikely that they play a role in its therapeutic actions. In rats Gheorgiu and his co-workers (1971) found that although the total volume of the gastric juices was decreased after carbenoxolone administration (100 mg/kg) the acid output was unchanged. In contrast (Henman, 1970) observed a reduction of total acidity in anaesthetised pylorus ligated rats.

Similarly, the results of studies in man are inconclusive. Only slight and insignificant changes in acid production have been noted by some authors after carbenoxolone treatment in normal subjects (Berstad et al, 1970; Ivey and Gray, 1973a) and in gastric ulcer patients (Bersted, 1972; Domschke et al, 1972). On the other hand Banks et al (1967) noted a reduction of 34% in maximal acid output after two weeks of therapy at a dose of 300 mg daily.

It is of interest to note that Rooney et al (1974) found higher levels of serum-immunoreactive secretin in patients treated with carbenoxolone. Secretin inhibits both the secretion and metabolic actions of gastrin, which itself is involved in mediating cell proliferation and stimulating acid secretion.
Back Diffusion of Hydrogen Ions

As with acid secretion reports on the effects of carbenoxolone on the back diffusion of hydrogen ions are conflicting. Colin-Jones and Taylor (1973) demonstrated that carbenoxolone treatment decreased ionic movement by about 30% in gastric ulcer patients. However, Ivey and Gray (1973a, b) could detect no significant influence of the drug on ionic fluxes in normal subjects receiving taurocholic acid or hydrochloric acid liquid meal tests. Their results, however, although not significant showed the same trends as those of Colin-Jones and Taylor (1973) where findings were obtained using a larger group.

Carbenoxolone has also been shown to prevent the significant increase in back diffusion caused by large doses of prednisolone (Domschke et al., 1975), and in dogs with Heidenhain pouches the back diffusion normally associated with bile injury was prevented (Cross and Rhodes, 1972; Calcraft et al., 1974).

Bile Reflux

Dippy (1973) in a study of 26 gastric ulcer patients found that carbenoxolone had no effect on the degree of bile reflux. This finding has now been challenged by Fisher and Larber (1975) who, using the more modern technique of measuring intraluminal potential difference and pyloric pressure, found that carbenoxolone may effect the degree of bile reflux in some patients.

Peptic Digestion

Berstad (1972) showed in both in vivo and in vitro studies that carbenoxolone was capable of reducing the peptic activity of human gastric juice. In vivo the inhibition was apparent for 40 to 60 minutes from the
time of dosing. Other studies in man have failed to confirm the results of Berstad, when oral rather than intragastric administration was used (Domschke et al., 1972a; Hausman and Tarnoky, 1966).

Walker and Taylor (1975a,b) established that successful carbenoxolone therapy was associated with a fall in total pepsin secretion in response to pentagastrin. They also noted a reduction in pepsin and an inability to sustain pepsin secretion.

In animal studies Henman (1970) observed a dose dependent inhibition of peptic activity by carbenoxolone. These results have been recently confirmed (Birnbaum and Karmeli, 1975). In this study carbenoxolone protected animals against the effects of taurocholic acid or aspirin and peptic activity was reduced by between 34% and 82%.

Thus it would appear that carbenoxolone induces a decrease in peptic activity in vitro and in vivo. This effect could be mediated in several ways:-
(1) Irreversible inactivation of pepsinogens in mucosal cells.
(2) Irreversible inactivation of pepsin in the lumen,
(3) Binding of pepsin in the lumen and thereby reducing its effective concentration.

This latter action is particularly feasible since pepsin is an albumin like protein, and the very strong affinity of carbenoxolone for albumin has been amply demonstrated (Parke and Lindup, 1973).

Mucosal Cell Turnover

The pathogenesis of the gastric ulcer involves an acceleration in the rate of DNA synthesis and epithelial cell turnover (Lipkin, 1971). In mice carbenoxolone treatment caused a 50% increase in the lifespan of mucosal cells and a corresponding decrease in the rate of DNA synthesis.
This effect has been confirmed in the human gastric mucosa (Croft, 1973; Klein et al, 1975). The number of mitoses decreased with treatment but the effect was limited to the ulcer regeneration zone and was not present in normal tissue.

**Mucus Synthesis**

Increased mucus production has been noted in patients following carbenoxolone treatment, both in gastric aspirates and surgical observations. This finding has been reported for normal subjects (Hausmann and Tarnoky, 1966) and for chronic gastric ulcer patients (Goodier et al, 1967, 1968). Goodier (1968) observed an abundance of PAS-positive mucosubstance after only 4 days of treatment. Neutral mucosubstance was adherent to both the gastric mucosa and to the crater of the healing ulcer.

This increase in gastric mucus synthesis after carbenoxolone treatment has been confirmed in studies on rats (Dean, 1968; Gheorgiu et al, 1972; Yeomans and St. John, 1974; Berg et al, 1975), guinea pigs (Lipkin and Ludwig, 1968), cats (Johnson, 1968) and dogs (Cross and Rhodes, 1972; Calcraft et al, 1974).

As well as increasing the volume of mucus secreted, there is evidence that carbenoxolone causes the production of a chemically modified glycoprotein (Domschke et al, 1972a,b). Parke (1972) has suggested that this could be mediated by the binding of the drug to functional proteins such as the nuclear histones. In man and rats Gheorgiu and his co-workers (1972) found that the N-acetylneuraminic acid (NANA) and hexosamine content of the gastric glycoprotein was elevated following carbenoxolone treatment.

Yeomans and St. John (1974) found no increase in the sialic acid content of rat gastric mucosal glycoprotein after carbenoxolone treatment.
but Domschke et al (1972a,b) found that in gastric ulcer patients NANA levels were depressed but became elevated after 4 weeks of carbenoxolone therapy. From these findings they proposed that NANA plays an important role in the protective function of mucus.

Following treatment of rats with carbenoxolone, Shillingford et al (1973) observed increased mucosal concentrations of hexosamines, fucose and sialic acids and an increase in the activity of gastric UDP-glucuronyl transferase. They also found (Shillingford et al, 1974) an increase in the rate of incorporation of N-acetylglucosamine, glucosamine, galactose, galactosamine, sialic acid and fucose into glycoproteins.

Further work by the same group (Johnston et al, 1975) has shown that the rate of incorporation of N-acetylglucosamine is markedly reduced in disease states such as gastric ulcer and gastritis. After treatment with carbenoxolone the clinical pattern of healing was accompanied by marked increases in the rate of incorporation.

It seems likely that gastric ulcer involves, at least in part, an impairment of the synthesis of glycoproteins necessary for the functioning of the gastric mucosal barrier. Carbenoxolone appears to exert a major action by restoring mucus synthesis to normal and perhaps modifying this synthesis to produce a more protective glycoprotein. The results of these alterations would inevitably be a stronger and more resistant gastric mucosal barrier.
1.4 AIM OF PROJECT

The aim of this project is to develop in vitro systems for studying the synthesis of gastric mucosal glycoprotein. To use these systems in determining the effects of ulcerogenic stimuli and anti-ulcerogenic drugs on mucus synthesis in human and animal gastric mucosae with a view to gaining insight into the pathogenesis of the gastric ulcer and the mode of action of anti-ulcer drugs. Furthermore, in view of the possible involvement of lysosomes in ulcerogenesis to study the effects of carbenoxolone sodium on lysosomal membrane stability.
CHAPTER TWO

GLYCOPROTEIN SYNTHESIS IN RAT, FERRET AND HUMAN GASTRIC MUCOSAE
2.1 INTRODUCTION

To understand the molecular interactions involved in the gelling and viscous properties of mucus, which are responsible for its protective and lubricating functions, requires a knowledge of its molecular components, their structure and behaviour. Glycoproteins, the principal constituents of mucus (Spiro, 1970), can be simply regarded as proteins which have carbohydrate covalently bound to their peptide core. They are widely distributed in nature, occurring not only in vertebrate and invertebrate animals but also in plants, unicellular organisms and viruses. The functions of these compounds are many and varied. As well as having a protective role they act as transport, immunoprotective, hormonal and structural agents in various tissues.

The amino acid composition of gastric mucosal glycoprotein is characterised by a high content of threonine, serine, proline, alanine and glycine and very low concentrations of aromatic and sulphur-containing amino acids. The composition of the "human principal gastric glycoprotein" (Schrager, 1969) is shown in Table 2.1.

The sugars which have been found in the carbohydrate portion of the mucous glycoproteins are N-acetylglucosamine, N-acetylgalactosamine, galactose, fucose and N-acetyl neuraminic acid (Schrager, 1969). The four main sugar components found in human gastric glycoprotein, N-acetylglucosamine, N-acetylgalactosamine, galactose and fucose exist in the ratio of 3:1:4:2 (Oates et al, 1974).

Attachment of the carbohydrate to the peptide portion of the glycoprotein involves a link between the C-1 carbon of the most internal sugar residue and a functional group of an amino acid within the peptide chain.
### Table 2.1 Composition of Principal Gastric Glycoprotein

<table>
<thead>
<tr>
<th>Components</th>
<th>Concentration (mol/mol glycoprotein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aspartic acid</td>
<td>52</td>
</tr>
<tr>
<td>Threonine</td>
<td>580</td>
</tr>
<tr>
<td>Serine</td>
<td>296</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>87</td>
</tr>
<tr>
<td>Proline</td>
<td>418</td>
</tr>
<tr>
<td>Glycine</td>
<td>120</td>
</tr>
<tr>
<td>Alanine</td>
<td>210</td>
</tr>
<tr>
<td>Valine</td>
<td>58</td>
</tr>
<tr>
<td>( \frac{1}{2} ) Cystine</td>
<td>trace</td>
</tr>
<tr>
<td>Methionine</td>
<td>0</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>41</td>
</tr>
<tr>
<td>Leucine</td>
<td>81</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>17</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>41</td>
</tr>
<tr>
<td>Lysine</td>
<td>29</td>
</tr>
<tr>
<td>Histidine</td>
<td>52</td>
</tr>
<tr>
<td>Arginine</td>
<td>52</td>
</tr>
<tr>
<td>Glucose</td>
<td>0</td>
</tr>
<tr>
<td>Mannose</td>
<td>0</td>
</tr>
<tr>
<td>Galactose</td>
<td>3596</td>
</tr>
<tr>
<td>Fucose</td>
<td>2376</td>
</tr>
<tr>
<td>Sialic acids</td>
<td>133</td>
</tr>
<tr>
<td>Glucosamine</td>
<td>2726</td>
</tr>
<tr>
<td>Galactosamine</td>
<td>928</td>
</tr>
<tr>
<td>Total hexosamine</td>
<td>3654</td>
</tr>
<tr>
<td>Total neutral sugar</td>
<td>5972</td>
</tr>
<tr>
<td>Sulphate</td>
<td>128</td>
</tr>
</tbody>
</table>

(From Schrager, 1969)
Because of the high proportion of serine and threonine in the peptide chain and similar number of N-acetylgalactosamine residues in the carbohydrate side chains it is believed that 'O' glycosidic linkages between these residues join the carbohydrate to the protein core. Moreover, studies employing α-N-acetylgalactosaminidase have shown this link to be α in anomeric configuration in hog gastric mucus (Weissmann and Henrichsen, 1969).

As yet the amino acid sequence around the amino acid residue involved in the glycosidic linkage has not been elucidated for mucus glycoproteins. In the case of other glycoproteins, e.g. immunoglobulins however where asparagine is the linking amino acid, the structure Asn-x-Thr(or Ser), where x is any amino acid is thought to be necessary for glycosylation to occur (Neuberger and Marshall, 1969). Such a structure may be necessary as a recognition sign for an enzyme attaching the first sugar residue of the carbohydrate chain.

Oates and his co-workers (1974) have studied the structure of the carbohydrate side chain of human gastric mucus glycoprotein carrying blood group H activity. Their studies indicate that a large number of carbohydrate chains (carrying numerous side chains of varying length) are attached to serine and threonine units of the protein through N-acetylgalactosamine residues. The carbohydrate chains are thought to be mainly alternating sequences of galactose linked to N-acetylglucosamine with fucose present at the end of the chain and also at branching points.

Fifty to seventy percent of the serine and threonine residues of the polypeptide chain are involved in glycosidic linkages (Tanaka and Pigman, 1965) indicating a very close spacing of carbohydrate chains along the polypeptide. In porcine submaxillary mucin there is an average of one carbohydrate chain per eight amino acid residues (Carlson, 1968). It is
believed that the close spacing of the carbohydrate groups containing negatively charged residues such as sialic acid may be responsible for the high viscosity of mucus (Gottschalk, 1960).

Interest in the chemistry and structure of glycoproteins has been stimulated by the demonstration that many mucus glycoproteins contain antigens, which in common with those of the erythrocyte cell surface belong to the A B H and Lewis blood group system (Watkins, 1972).

Foremost among the blood group active glycoproteins examined have been those isolated from ovarian cyst fluid in which it is evident that blood group activity is determined by terminal sugar substituents in the form of galactose, N-acetylgalactosamine and fucose linked to the carbohydrate chain core (Watkins, 1966). Figure 2.1 shows the structures of the immunological determinants of A B H and Lewis blood group activities in ovarian cyst glycoprotein. These structures have been confirmed by the work of Oates et al (1974) for human gastric glycoprotein.

It is of interest to note that while sialic acid substituents occur as terminal substituents in the carbohydrate of A B H and Lewis blood group active glycoproteins this sugar does not have an antigenically determining role and indeed its removal often permits enhanced reactivity (Baig and Aminoff, 1972).

The presence of a sulphated glycoprotein has been established in the hog gastric mucosa (Slomiany and Meyer, 1972; Liau and Horowitz, 1974). This glycoprotein, which possesses both A and H blood group activities is sulphated by an ester link through N-acetylgalcosamine. The structure of the sulphated carbohydrate chain of this glycoprotein has been
Figure 2.1  Structure of the immunological determinants of
ABH and Lewis blood group activities (Watkins, 1966)

<table>
<thead>
<tr>
<th>Blood Group</th>
<th>Structure</th>
</tr>
</thead>
<tbody>
<tr>
<td>H</td>
<td>Gal $\beta^{1-3(4)}$ GlcNAc $\alpha 1-2$ Fuc</td>
</tr>
<tr>
<td>A</td>
<td>GalNAc $\alpha 1-3$ Gal $\beta^{1-3(4)}$ GlcNAc $\alpha 1-2$ Fuc</td>
</tr>
<tr>
<td>B</td>
<td>Gal $\alpha 1-3$ Gal $\beta^{1-3(4)}$ GlcNAc $\alpha 1-2$ Fuc</td>
</tr>
<tr>
<td>Le$^a$</td>
<td>Gal $\beta^{1-3}$ GlcNAc $\alpha 1-4$ Fuc</td>
</tr>
<tr>
<td>Le$^b$</td>
<td>Gal $\beta^{1-3}$ GlcNAc $\alpha 1-2$ Fuc $\alpha 1-4$ Fuc</td>
</tr>
</tbody>
</table>
determined by Slomiany and Meyer (1973) and is shown in Figure 2.2. The presence of endogenous sulphated glycoprotein in human gastric mucus has not, however, been positively demonstrated (Lambert and Andre, 1972).

Little is known of the secondary structure of gastric mucus although it is likely that they have a 'bottle brush' structure similar to that proposed for the blood group substances (Morgan, 1959) with a core of protein and side chains of carbohydrate. This concept is supported by the work of Bettleheim (1963) whose infra-red studies on bovine submaxillary glycoprotein demonstrated that the carbohydrate side chains appeared to be at right angles to the polypeptide.

Allen and Snary (1972) have studied the structure of glycoproteins of soluble mucus from the cardiac region of the pig stomach. Two glycoprotein fractions, A (molecular weight $2 \times 10^6$) and B (molecular weight $1.1 \times 10^5$) were isolated. Similarities in chemical analysis, blood group activities (A and H) and biosynthetic patterns (Snary and Allen, 1971, 1972) led these authors to conclude that low molecular weight glycoprotein B was a repeating unit of glycoprotein A, this molecule being a discrete assembly of about 18 glycoprotein B units joined covalently. Furthermore treatment of glycoproteins A and B with mercaptoethanol, which breaks disulphide bridges split both glycoproteins into four subunits of molecular weight $5.2 \times 10^5$ for glycoprotein A and $2.8 \times 10^4$ for glycoprotein B (Starkey et al., 1974).

Thus a picture of the highly polymeric structure of mucoproteins, necessary for their viscous properties, can be obtained. The basic
Figure 2.2  Structure of carbohydrate side chain from hog gastric sulphated glycoprotein
(Slomiany and Meyer, 1973)

Blood Group A activity

GalNAc $\alpha 1-3$ Gal $\beta 1-3(4)$ GlcNAc $\beta 1-4$ Gal

$\alpha 1-2$

Blood Group H activity

Gal $\beta 1-3(4)$ GlcNAc $\beta 1-4$ Gal

Fuc $\alpha 1-2$

Gal $\beta 1-4$ GlcNAc (?) $\beta 1-4$ GlcNAc $\beta 1-3$ Gal

6-sulphate

GalNAc $\beta 1-6(3)$

Gal $\beta 1-3(4)$

serine or threonine
A subunit of the glycoprotein has a molecular weight of about 28,000. Four of these units are joined by disulphide bridges to form the low molecular weight mucoprotein B of the water soluble mucus. Mucoprotein B in turn is polymerised to form the high molecular weight mucoprotein A in such a way that A is also composed of four subunits joined by disulphide linkages. A diagrammatic representation of this molecule is shown in Figure 2.3.

**Biosynthesis of glycoproteins**

It has been concluded from studies using classical inhibitors of protein synthesis (Spiro and Spiro, 1966), and from the autoradiographical demonstration of localization of growing polypeptide chains around the polysomes (Neutra and Leblond, 1966), that biosynthesis of the protein fraction of the glycoprotein takes place before and occurs independently of carbohydrate attachment.

Once incorporated into the polypeptide chains of mucus glycoprotein the amino acids serine and threonine may be glycosylated.

The addition of the first carbohydrate unit to the peptide chain and subsequent elongation of the carbohydrate chain is affected by a group of enzymes known as the glycosyl transferases. These enzymes catalyse the transfer of sugar residues from sugar nucleotide donors, (UDP-galactose, UDP N-acetylglucosamine, UDP N-acetylgalactosamine, CMP-N-acetylneuraminic acid and GDP fucose) to the polypeptide or growing carbohydrate chains as the glycoprotein moves from the rough endoplasmic reticulum to the smooth endoplasmic reticulum and Golgi complex. Evidence exists that glycosylation may occur even before the polypeptide chain has left the ribosome (Lawford and Schachter, 1967; Louisot et al, 1967).
Fig. 2.3 A diagramatic representation of the four glycoprotein subunits joined to form the gastric glycoprotein.
Experimental evidence suggests that differences occur in the sub-cellular distribution of the glycosyltransferases. Thus Bouchilloux et al. (1970) found that the rough endoplasmic reticulum of sheep thyroid is enriched in enzymes required to transfer the internal sugars, N-acetylglucosamine and mannose, whereas the Golgi-rich fractions could transfer galactose and N-acetylgalactosamine to more external positions of thyroglobulin precursors. Similarly, all three enzymes that catalyse the transfer of sialic acid, galactose and N-acetylgalactosamine to form the terminal trisaccharide of plasma proteins have been found to be concentrated in the Golgi fraction of rat liver (Schachter, et al., 1970).

The exact mechanism involved in determining completion of the polysaccharide chain and transportation of the glycoprotein molecule remains unknown. It has been proposed that the addition of a specific sugar residue (fucose, NANA) or of a specific steric configuration such as the α-D-configuration of N-acetylgalactosamine and D-galactose in human blood group A and B substances respectively may stop any further incorporation of sugar components (Gottschalk, 1969).

Little is known of the different phenomena contributing to the regulation of glycoprotein biosynthesis. Whereas synthesis of the protein core is under direct genetic control, glycosylation is not. It is subject to the influence of enzyme specificity and substrate and cofactor availability. The various stages of carbohydrate attachment therefore stand out as logical points for rapid physiological control and could also be the loci where pathological processes could operate to influence the rate of synthesis or the nature of the product formed.

The high specificity of the glycosyl transferases, towards both the sugar nucleotide and the acceptor, permits the synthesis of complex carbohydrate units with structures defined in regard to sequence and
branching as well as position and anomeric configuration of the linkages. Acceptor specificity has been shown for a fucosyltransferase from rat small intestinal mucosa (Bella and Kim, 1971). This enzyme was shown to be active in transferring fucose to a terminal galactose residue of a glycoprotein or oligosaccharide when galactose was linked by a β-glycosidic bond to the next sugar. Similarly, Tuppy and Schenkel-Brunner (1969) found that the requirements for N-acetylgalactosaminyltransferase and galactosyltransferase from pig stomach were identical except in their donor specificities, one requiring UDP N-acetylgalactosamine and the other UDP-galactose.

Although regulation of glycosylation is not under nucleic acid template control it is under genetic control insofar as the synthesis of the transferase enzymes is template controlled. Thus α-D galactosyltransferase which transfer D-galactose in α linkage to the terminal galactose of blood group H active glycoproteins, conferring group antigenicity were found to be absent in tissues from A or O group donors (Poretz and Watkins, 1972), implying that the gene coding for this enzyme is absent or inoperative in these groups. It is of interest to note that the incidence of gastric ulcer is high among blood group O subjects.

It has also been suggested that the subcellular distribution of the glycosyltransferases could play a role in the control of glycoprotein synthesis (Schachter, 1974). Thus the localisation of N-acetylneuraminyltransferase in the Golgi complex would preclude N-acetylneuraminic acid from appearing in the carbohydrate chain anywhere other than in terminal positions (Schachter et al, 1970).
Catabolism of glycoproteins is thought to occur mainly in the lysosomes (Aronson and De Duve, 1968; Mahadevan et al., 1969). These subcellular organelles contain glycosidases specific for almost every sugar and anomeric configuration occurring in glycoproteins (Patel and Tappel, 1971). The breakdown of glycoproteins by lysosomal hydrolases is discussed in Chapter 4.

The precise means by which gastric mucus exerts its protective action is unknown. The high viscosity of mucus has been accepted as an important protective feature (Rainsford, 1975). This high viscosity has been attributed to the ability of the mucus glycoproteins to form high molecular weight complexes (Starkey et al., 1974), to the close spacing of the carbohydrate chains along the polypeptide core (Carlson, 1968) and to the presence of acidic groups, such as sialic acid and possibly sulphate moieties (Gottschalk and Thomas, 1961). Domschke and his co-workers (1972) found NANA levels depressed in mucus from gastric ulcer patients.

As well as a possible role maintaining the viscosity of gastric mucus it has been suggested that sulphated glycoproteins may protect the mucosa through an anti-peptic effect (Prino et al., 1972).

The Effects of Drugs and Disease States on the Synthesis of Mucus

Histological, biosynthetic and chemical analysis studies have demonstrated that pathological conditions and drug therapy may be associated with changes in the amount of mucus secreted and/or in the quantitative relationships of glycoprotein components.

Of the drugs associated with gastric damage the most widely studied with respect to mucus synthesis are aspirin and sodium salicylate. A
review of the biochemical pathology of aspirin-induced gastric damage has recently been published (Rainsford, 1975). Histochemical studies and chemical analyses have shown that repeated administration of salicylates results in a reduction in the quantity of the sulphate and carbohydrate components of gastric mucus in the rat (Menguy and Masters, 1965; Ganter et al, 1966; Johannson and Linquist, 1970) and dog (Hakkinen et al, 1968; Lev et al, 1972). Moreover, biosynthetic studies have shown aspirin to inhibit the uptake of $[^14C]$glucose (Kent and Allen, 1968) and $^{35}$SO$_4^{--}$ (Rainsford, 1975).

Phenylbutazone has been shown to cause changes in the carbohydrate to protein ratios in the dog stomach, with a decrease in the sialic acid, fucose and hexosamine content of the mucus (Menguy and Desbaillets, 1967). Similar findings are reported for the rat stomach (Takagi and Abe, 1974). Similar results have also been obtained for cortisone (Menguy and Masters, 1963) and ACTH (Desbaillets and Menguy, 1967) in dog stomach and for prednisolone (Roberts and Mezami, 1963) in the rat.

Other factors having an adverse effect on the gastric mucosa include stress due to cold, water immersion, restraint stress (Takagi and Yano, 1972) and starvation (Menguy, 1969). These have been shown to decrease the hexosamine content of rat gastric mucus and Lambert et al (1970) have shown a decrease in the incorporation of radiosulphate in restrained rats.

In pathological conditions of the human gut alterations in mucus synthesis have also been observed. In ulcerative colitis there is a significant increase in mannose containing glycoprotein (Teague et al, 1973) and mucus.glycoprotein from gastric ulcer patients has a significantly reduced N-acetyleneuraminic acid content (Domschke et al, 1972).
Gastric carcinoma is associated with an alteration in the quantitative relationships between the sugar components of the carbohydrate chains of mucus glycoprotein (Schrager and Oates, 1973). These authors also found a change in blood group specificity of the glycoproteins from cancerous cells compared with that of the hosts red blood cells.

The role of sulphated glycoproteins in human gastric mucus is a subject of controversy. Lambert and Andre (1972) have suggested that unlike the dog (Ley et al., 1969), pig and rat (Rainsford, 1975) the normal human adult gastric mucosa does not secrete sulphated glycoproteins and that their presence in gastric juice is due to contamination by saliva and bronchial secretions. In pathological conditions such as gastric carcinoma, however, the situation appears to be different and sulphated glycoproteins are secreted both by the tumour and surrounding tissue (Lev, 1970; Gad, 1969).

Modifications in the synthesis and secretion of glycoproteins may also be affected by drugs which are beneficial in the treatment of gastric lesions. Carbenoxolone sodium (Goodier, 1967, 1968) the prostaglandin analogue 15(R)15 methyl-E2-methyl ester (Fung et al., 1974), zolimidine (Celli et al., 1975), glutamine, gefarnate and sodium copper chlorphylline (Takagi and Yano, 1972) have been shown to increase the rate of synthesis of gastric mucin. Moreover, carbenoxolone has been shown to increase the amount of protein bound hexosamines and sialic acids in mucins from rat and human stomachs (Shillingford et al., 1973; Gheorghiu et al., 1972; Domschke et al., 1972).

This chapter contains details of studies carried out to determine the effects of the anti-ulcerogenic drug, carbenoxolone and a mucosal damaging drug, ethanol, on glycoprotein synthesis in rat and ferret
stomachs, glycoprotein synthesis being measured by the rate of uptake of radiolabelled precursors into an acid precipitable fraction of gastric discs. Using this same system the effects of drugs and disease on glycoprotein synthesis has been studied in the human stomach.
2.2 MATERIALS AND METHODS

Chemicals

Carbenoxolone sodium was a gift from Biorex Laboratories Ltd., London. All radiochemicals used (Table 2.2) were obtained from the Radiochemical Centre, Amersham. Scintillation chemicals were purchased from the Packard Instrument Company. All other chemicals were Analar grade wherever possible and were supplied by either British Drug Houses Ltd., Poole, or the Sigma Chemical Co., London.

Animals

Female Wistar albino rats (200 g), derived from the Porton strain were developed at the Laboratory Animals Centre, Carshalton, and bred in the University of Surrey Animal Unit. The animals were housed in groups of three to six and were allowed food (Spratts Laboratory Diet No. 1) and water ad libitum, except where specified.

Male ferrets (approximately 1 kg) were purchased from reputable dealers for the study. The animals were housed in pairs in the University of Surrey Animal Unit. They were fed on a diet of 'Bounce' dog food, dead mice and milk and were allowed water ad libitum.

Incorporation of Radiolabelled Precursors into Acid Precipitable Fraction of Gastric Discs

The technique used was that of Lukie and Forstner (1972) as modified for gastric tissue by Shillingford (1975). Rats were killed by cervical dislocation and the stomachs immediately placed in ice cold KCl (1.15% W/v). Each stomach was opened along the greater curvature and washed free of its contents with ice cold saline. Stomach resections were taken from the pyloric antrum using a 14 mm diameter cork borer, the stomach
<table>
<thead>
<tr>
<th>Compound</th>
<th>Specific Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>N-acetyl-D-[1-3H]glucosamine</td>
<td>4.0 Ci/mmol</td>
</tr>
<tr>
<td>D-[6-3H]glucosamine</td>
<td>12.6 Ci/mmol</td>
</tr>
<tr>
<td>D-[1-14C]galactose</td>
<td>59.0 mCi/mmol</td>
</tr>
<tr>
<td>D-[1-3H]fucose</td>
<td>3.61 Ci/mmol</td>
</tr>
<tr>
<td>N-acetyl-[4,5,6,7,8,9-14C]neuraminic acid</td>
<td>262.0 mCi/mmol</td>
</tr>
<tr>
<td>L-[G-3H]proline</td>
<td>500.0 mCi/mmol</td>
</tr>
<tr>
<td>L-[3-3H]serine</td>
<td>5.3 Ci/mmol</td>
</tr>
<tr>
<td>L-[G-3H]asparagine</td>
<td>100.0 mCi/mmol</td>
</tr>
<tr>
<td>L-[G-14C]threonine</td>
<td>232.0 mCi/mmol</td>
</tr>
<tr>
<td>Sodium-[35C]sulphate</td>
<td>70.6 mCi/mmol</td>
</tr>
<tr>
<td>D-[1-3H]galactose</td>
<td>5.2 Ci/mmol</td>
</tr>
</tbody>
</table>
first being gently stretched out on a cork dissection board. A similar procedure was used in the preparation of discs from the ferret stomach. These animals were killed by CO₂ suffocation rather than cervical dislocation.

Stomach discs were placed in 20 ml stoppered test tubes containing incubation medium, and preincubated for 10 min at 37°C in a Mickle shaking incubator at 100 cycles/min. Incorporation was begun by the addition of labelled precursor (0.25-2.5 μCi in 0.1 ml 0.9% w/v NaCl). The radiolabelled materials were used as follows: N-acetyl[^3]H]glucosamine, 1 μCi (2.5 μCi in ethanol study);[^3]H]glucosamine, 1 μCi;[^14]C]galactose, 0.5 μCi; N-acetyl[^14]C]neuraminic acid, 0.25 μCi;[^3]H]fucose, 0.5 μCi; amino acids, 0.5 μCi[^35]S]Na₂SO₄, 1 μCi.

The final volume of medium was 2 ml and contained 236 μmol, NaCl; 11.2 μmol KCl; 6.0 μmol CaCl₂; 2.8 μmol KH₂PO₄; 2.8 μmol MgSO₄, 58.6 μmol NaHCO₃; and 16 μmol l-glutamine. Tubes were flushed with O₂-CO₂ (95-5 V/V) every 10 min.

Incubation periods were between 1.5h and 6h and were terminated by draining the medium and washing the gastric discs twice with ice cold saline.

Isolation of the Glycoprotein Fraction

(i) Acid precipitation. Washed gastric sections were homogenised in 5 mM EDTA (pH 7.4, 20 ml) with a Polytron homogeniser and glycoproteins were precipitated by storage at 4°C after addition of an equal volume of trichloroacetic acid: phosphotungstic acid mixture (20% w/v: 2% w/v). The acid precipitated fraction was collected by centrifugation at 200 g for 10 min. The precipitate was washed twice with distilled water and contaminating lipid material removed by two extractions with chloroform-methanol (1:1 V/V). The material was then air dried.
(ii) Cetylpyridinium chloride precipitation. Washed gastric sections were homogenised in 10 ml distilled water using a Polytron homogeniser. The homogenates were then digested by twice-crystallised pepsin (2 mg/ml) at pH 1.6 for 24h at 37°C. The pH was then increased to 7.5 by the addition of 1M-NaOH and glycoproteins precipitated by the addition of cetylpyridinium chloride (final concentration 1% w/v). The precipitate so formed was allowed to aggregate overnight at pH 7.5 at room temperature and separated by centrifugation. The precipitate was washed, extracted for lipids and dried as described above.

Determination of Radioactivity. The dried glycoprotein fractions were weighed into scintillation vials and suspended in distilled water (0.5 ml). Perchloric acid (0.3 ml, 60% w/v) was added, followed after a 10 min interval by hydrogen peroxide (0.6 ml, 100 vols). The vials were then sealed with plastic liners and screw caps and were heated at 70°C for 90 min with occasional agitation. On cooling 15 ml scintillator (toluene-Triton X-100, 2:1 v/v; PPO 0.55% w/v) was added to each vial which was then thoroughly mixed.

Scintillation counting of radioactivity was carried out using a Packard Tri-Carb Spectrometer (Model 3320) using the internal standardization method for determining counting efficiency.

Examination of the Effects of Drugs. The effects of drugs in vivo on the in vitro incorporation system were determined by pretreatment of animals with the drug in a suitable vehicle, control animals receiving vehicle only. Details of the compounds, dose levels and length of the dosing period are given in the 'Experimental Results' section.
Incorporation of Radiolabelled Sugar into Human Gastric Mucosal Glycoprotein

Human gastric biopsy samples were obtained during gastroscopic investigation of patients showing symptoms of gastrointestinal disorder. The endoscopic procedure was as follows: Patients were given a pre-operative dose of Buscopam (hyoscine N-butylbromide) to reduce secretion and relax gastrointestinal musculature, and were anaesthetised by intravenous injection of Valium until amnesic but semi-conscious. The pharynx having been anaesthetised with a lignocaine spray the endoscope was eased into the oesophagus and gently pushed through into the stomach. Tissue biopsy samples were obtained using controlled forceps which removed approximately 100 mg of tissue. Two such biopsies were taken from the lesser curve, greater curve and antrum, from each stomach.

The samples were placed immediately in stoppered tubes containing 2 ml of incubation medium at 0°C and stored in an ice bath until incubation could be carried out. The time between sampling and incubation was never more than two hours.

The incorporation assay used was identical to that used for gastric sections. The small tissue samples after incubation being homogenised in 5.0 ml EDTA (5 mM, pH 7.4) and glycoproteins precipitated by the addition of 5 ml trichloroacetic acid-phosphotungstic acid (20\%:2\% w/v).

True normal samples from volunteer subjects were not obtained for ethical reasons. Instead 'apparent normals' i.e. samples from those patients with macroscopically normal stomachs as viewed through the endoscope, were used.
2.3 EXPERIMENTAL AND RESULTS

Using this incorporation system it was shown that after an initial lag phase the uptake of N-acetylglucosamine into the acid precipitated fraction was linear at least until the fourth hour of incubation (Figure 2.3), could be inhibited by cycloheximide ($10^{-4} M$), a classical inhibitor of protein synthesis (Figure 2.3) and had an optimum N-acetylglucosamine concentration of 25 mM (Figure 2.4). However, it was decided to work at sub-optimal concentrations of substrate since achievement of optimal concentrations by dilution of labelled substrate with unlabelled substrate reduced the specific activity of the precipitated glycoproteins to an unacceptable level (Figure 2.5).

Effect of Carbenoxolone on the Incorporation of Labelled Precursors into Rat Gastric Discs

The incorporation of N-acetyl-$[^3]H$glucosamine, $[^3]H$serine,$[^14]C$ threonine and $^{35}SO_4^{2-}$ into the precipitable fraction of gastric discs from rats treated for 7 days with carbenoxolone (25 mg/kg/day) was carried out. Control animals received drinking water only. The results of these studies are shown in Table 2.3.

Carbenoxolone pretreatment had a significant effect on the uptake of N-acetylglucosamine, an increase of 71% over the controls being observed but did not cause a significant change in the incorporation rate of the amino acids, serine and threonine. There was, however, a trend towards an increase in uptake particularly of threonine.

Significant changes in the rate of incorporation of sodium$[^35]S$ sulphate were seen, both in the fraction precipitated by acid (100% increase) and cetylpyridinium chloride (CPC) (110% increase). The
Incorporation of N-acetylglucosamine

Fig. 2.3 Effect of cycloheximide (10^{-4}M) on the incorporation of N-acetylglucosamine into an acid precipitable fraction of rat gastric discs.

Results are means ± SEM of four samples. Closed circles represent control discs, open circles treated discs.
Fig. 2.4 Effect of N-acetylglucosamine concn on the incorporation of N-acetyl-[\textsuperscript{3}H]glucosamine into an acid precipitable fraction of rat gastric discs.

Results are the means ± SEM of four observations.

Fig. 2.5 Effect of N-acetylglucosamine concn on the incorporation of N-acetyl-[\textsuperscript{3}H]-glucosamine into an acid precipitable fraction of rat gastric discs.

Results are the means ± SEM of four observations.
Table 2.3 The Effects of Carbenoxolone on the Rate of Incorporation of Radiolabelled Precursors into a Precipitable Fraction of Rat Gastric Discs

<table>
<thead>
<tr>
<th>Precursor</th>
<th>Method of precipitation</th>
<th>Incorporation rate mmol x 10^{-12}/mg glycoprotein/h</th>
<th>Change from control %</th>
</tr>
</thead>
<tbody>
<tr>
<td>N-acetyl-[^{3}H]glucosamine</td>
<td>Acid</td>
<td>$32 \pm 8 \ (6)$</td>
<td>$55 \pm 6 \ (6)^* \ + \ 71%$</td>
</tr>
<tr>
<td>[{^{3}}H]serine</td>
<td>Acid</td>
<td>$62 \pm 9 \ (5)$</td>
<td>$74 \pm 13 \ (5)\ N.S. \ + \ 19%$</td>
</tr>
<tr>
<td>[{^{14}}C]threonine</td>
<td>Acid</td>
<td>$1260 \pm 172 \ (5)$</td>
<td>$1990\pm440 \ (5)\ N.S. \ + \ 57%$</td>
</tr>
<tr>
<td>[{^{35}}S]-Na_{2}S_{4}</td>
<td>Acid</td>
<td>$7 \pm 1 \ (6)$</td>
<td>$14 \pm 1 \ (6)^{**} \ +100%$</td>
</tr>
<tr>
<td>[{^{35}}S]-Na_{2}S_{4}</td>
<td>CPC</td>
<td>$3609 \pm 473 \ (6)$</td>
<td>$7590\pm205 \ (6)^{**} \ +\ 110%$</td>
</tr>
</tbody>
</table>

Results are expressed as means ± S.E.M. of the number of animals shown in parentheses.

Students t-test * P < 0.05, ** P < 0.01, N.S. not significant.
rate of incorporation into the fraction precipitated by CPC was very much greater than that of the acid precipitation by several orders of magnitude.

**Effect of Carbenoxolone on the Incorporation of Radio-labelled Precursors into an Acid-Precipitable Fraction of Ferret Gastric Discs**

Ferrets were dosed with carbenoxolone (25 mg/kg/day) in their drinking water for seven days. Control animals received water only. After seven days the animals were sacrificed and the incorporation assays carried out. The effects of carbenoxolone on the uptake of sugar and amino acid precursors are shown in Tables 2.4 and 2.5 respectively.

As with the rat, carbenoxolone failed to show any significant effect on the incorporation of amino acids in the ferret stomach. Again, however, there was a trend towards an increased uptake, particularly of serine in this species. A significant increase was observed for all of the sugars investigated with the exception of galactose for which the incorporation rate remained unchanged. The largest increase occurred with N-acetylglucosamine the incorporation rate of which was increased by approximately 250% over the controls.

**The Effect of Ethanol on the Incorporation of N-acetyl[^3]H]glucosamine into Rat Gastric Resections**

Rats were killed one hour after dosing with ethanol (1 ml, 35% V/v aqueous soln. 1.9 mg/kg) by intubation and the incorporation of N-acetyl[^3]H]glucosamine (2.5 μCi) measured. Control rats received water (1 ml) only. A note was made of rats whose stomachs showed haemorrhagic areas. The results are shown in Table 2.6.
Table 2.4 The Effects of Carbenoxolone in the Rate of Incorporation of Sugar Precursors into an Acid Precipitable Fraction of Ferret Gastric Discs

<table>
<thead>
<tr>
<th>Precursor</th>
<th>Incorporation rate mmol x 10^-12/mg glycoprotein/h</th>
<th>Change from control %</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Carbenoxolone-treated</td>
</tr>
<tr>
<td>N-acetyl-[(^3\text{H})] glucosamine</td>
<td>140 ± 4 (3)</td>
<td>480 ± 18 (6) *</td>
</tr>
<tr>
<td>(^3\text{H})glucosamine</td>
<td>4 ± 0.4 (3)</td>
<td>7 ± (0.9) (6) *</td>
</tr>
<tr>
<td>(^{14}\text{C})galactose</td>
<td>3300 ± 410 (3)</td>
<td>3400 ± 670 (6) N.S.</td>
</tr>
<tr>
<td>N-acetyl-[(^{14}\text{C})] neuraminic acid</td>
<td>275 ± 20 (3)</td>
<td>370 ± 30 (6) *</td>
</tr>
<tr>
<td>(^3\text{H})fucose</td>
<td>1.7 ± 0.03(6)</td>
<td>2.2 ± 0.04(6) *</td>
</tr>
</tbody>
</table>

Results are expressed as means ± S.E.M. of the number of animals shown in parentheses.

Students 't' test * P < 0.05, N.S. not significant.
Table 2.5 The Effects of Carbenoxolone on the Rate of Incorporation of Amino Acids into an Acid Precipitable Fraction of Ferret Gastric Discs

<table>
<thead>
<tr>
<th>Precursor</th>
<th>Incorporation rate mmol x 10^-12/mg glycoprotein/h.</th>
<th>Change from control %</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Carbenoxolone-treated</td>
</tr>
<tr>
<td>(^{14}\text{C}) threonine</td>
<td>560 ± 54 (3)</td>
<td>600 ± 22 (3) N.S.</td>
</tr>
<tr>
<td>(^3\text{H}) serine</td>
<td>63 ± 8 (3)</td>
<td>123 ± 40 (3) N.S.</td>
</tr>
<tr>
<td>(^3\text{H}) proline</td>
<td>60 ± 23 (3)</td>
<td>45 ± 23 (3) N.S.</td>
</tr>
<tr>
<td>(^3\text{H}) asparagine</td>
<td>126 ± 45 (3)</td>
<td>122 ± 32 (3) N.S.</td>
</tr>
</tbody>
</table>

Results are expressed as means ± S.E.M. for the number of observations shown in parentheses.

Students 't' test. N.S. not significant.
Table 2.6  The Effect of Alcohol Pretreatment on the Incorporation of $N$-acetyl-$[^3]H$]glucosamine into an Acid Precipitable Fraction of Rat Gastric Discs

<table>
<thead>
<tr>
<th>Incorporation Rate</th>
<th>Presence of Haemorrhagic Areas</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\text{mmol} \times 10^{-12}/\text{mg glycoprotein}$</td>
<td></td>
</tr>
<tr>
<td>Control animals</td>
<td>Alcohol treated</td>
</tr>
<tr>
<td>1 143</td>
<td>2 2</td>
</tr>
<tr>
<td>2 164</td>
<td>10 +</td>
</tr>
<tr>
<td>3 277</td>
<td>45 +</td>
</tr>
<tr>
<td>4 89</td>
<td>6 +</td>
</tr>
<tr>
<td>5 85</td>
<td>51 -</td>
</tr>
<tr>
<td>6 276</td>
<td>58 -</td>
</tr>
</tbody>
</table>

Mean + S.E.M. 172 + 26 29 + 11

Students $t$-test $p < 0.001$. 
Alcohol had a potent inhibitory effect on the uptake of the sugar into the glycoprotein fraction, the rate of incorporation of N-acetyl $[^3\text{H}]$glucosamine being reduced by 83% of the control rate. Also in four of the six rats treated with alcohol haemorrhagic areas could be seen in the stomach.

**Incorporation of N-acetyl$[^3\text{H}]$glucosamine and $[^3\text{S}]$ Sodium Sulphate into an Acid Precipitable Fraction of Human Gastric Biopsy Samples**

The incorporation of labelled precursors into glycoproteins of human biopsy samples was carried out by the methods outlined in the Materials and Methods section. Substrates used in these studies were N-acetyl$[^3\text{H}]$ glucosamine (1 $\mu$Ci) and sodium $[^3\text{S}]$ sulphate (1 $\mu$Ci). Biopsy samples were obtained from apparently normal stomachs, diseased stomachs and in a few cases from the stomachs of patients with gastric ulcer, before and after carbenoxolone treatment. The results of these studies are shown in Tables 2.7 - 2.11.

Table 2.7 shows that in the normal stomach the incorporation rate of N-acetylglucosamine varies from one anatomical section of the stomach to another. Thus the antral mucosa has a higher rate of uptake of the sugar than does the lesser and greater curves which have approximately equal rates of incorporation.

Markedly different incorporation rates were seen in biopsy samples taken from diseased areas of the stomach [Table 2.8(i)]. Thus the uptake of N-acetylglucosamine into lesser curve biopsies from stomachs bearing an ulcer in this area is significantly reduced. A similar trend was observed in biopsies from antral mucosa in the same stomachs. In this case however, the trend was not significant. Incorporation into greater curve samples was not effected.
Table 2.7

Incorporation of N-acetyl-[3H]glucosamine into an Acid Precipitable Fraction of Biopsy Samples from Apparently Normal Human Stomachs

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>Incorporation Rate mmol x 10^{-12}mg glycoprotein/h</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pyloric Antrum</td>
</tr>
<tr>
<td>1</td>
<td>211</td>
</tr>
<tr>
<td>2</td>
<td>226</td>
</tr>
<tr>
<td>3</td>
<td>304</td>
</tr>
<tr>
<td>4</td>
<td>227</td>
</tr>
<tr>
<td>5</td>
<td>117</td>
</tr>
<tr>
<td>6</td>
<td>300</td>
</tr>
<tr>
<td>7</td>
<td>315</td>
</tr>
<tr>
<td>8</td>
<td>284</td>
</tr>
<tr>
<td>9</td>
<td>192</td>
</tr>
<tr>
<td>10</td>
<td>175</td>
</tr>
<tr>
<td>11</td>
<td>214</td>
</tr>
<tr>
<td>12</td>
<td>253</td>
</tr>
<tr>
<td>13</td>
<td>207</td>
</tr>
<tr>
<td>14</td>
<td>278</td>
</tr>
<tr>
<td>15</td>
<td>289</td>
</tr>
<tr>
<td>16</td>
<td>220</td>
</tr>
<tr>
<td>17</td>
<td>238</td>
</tr>
<tr>
<td>18</td>
<td>229</td>
</tr>
<tr>
<td>19</td>
<td>269</td>
</tr>
<tr>
<td>20</td>
<td>199</td>
</tr>
<tr>
<td>21</td>
<td>152</td>
</tr>
<tr>
<td>22</td>
<td>273</td>
</tr>
<tr>
<td>23</td>
<td>236</td>
</tr>
<tr>
<td>24</td>
<td>220</td>
</tr>
</tbody>
</table>

Means ± S.E.M 234 ± 9.8 148 ± 9.6 151 ± 9.5

Students' t-test

- Pyloric antrum v Greater curve  P < 0.01
- Pyloric antrum v Lesser curve  P < 0.01
- Greater curve v Lesser curve  Not significant
Table 2.8 Incorporation of N-acetyl-\(^3\)H\)glucosamine into Acid Precipitable Fractions of Human Gastric Biopsy Samples in Diseased States of the Stomach

(i) Ulcer on lesser curve

<table>
<thead>
<tr>
<th>Patient (age)</th>
<th>Clinical condition</th>
<th>Incorporation Rate mmol x 10(^{-12})/mg glycoprotein/h.</th>
<th>Antrum</th>
<th>Lesser Curve</th>
<th>Greater Curve</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male, 64</td>
<td>Ulcer on lesser curve near pylorus</td>
<td>-</td>
<td>66</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male, 73</td>
<td>Ulcer on lesser curve</td>
<td>-</td>
<td>33</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female, 48</td>
<td>Ulcer on lesser curve</td>
<td>244</td>
<td>95</td>
<td>116</td>
<td></td>
</tr>
<tr>
<td>Female, 64</td>
<td>Ulcer on lesser curve</td>
<td>165</td>
<td>99</td>
<td>116</td>
<td></td>
</tr>
<tr>
<td>Female, 65</td>
<td>Ulcer on lesser curve</td>
<td>223</td>
<td>125</td>
<td>319</td>
<td></td>
</tr>
<tr>
<td>Female, 52</td>
<td>Ulcer on lesser curve</td>
<td>221</td>
<td>113</td>
<td>160</td>
<td></td>
</tr>
<tr>
<td>Male, 53</td>
<td>Ulcer on lesser curve</td>
<td>232</td>
<td>105</td>
<td>147</td>
<td></td>
</tr>
<tr>
<td>Male, 54</td>
<td>Ulcer on lesser curve</td>
<td>193</td>
<td>87</td>
<td>122</td>
<td></td>
</tr>
</tbody>
</table>

Means \(\pm\) S.E.M. 213 \(\pm\) 13.0 N.S. 90.2 \(\pm\) 10.1** 163 \(\pm\) 35 N.S.

Normal means \(\pm\) S.E.M.(Table 2.7) 234 \(\pm\) 9.8 148 \(\pm\) 9.6 151 \(\pm\) 9.5

Students' t-test Ulcerated lesser curve v normal** \(P < 0.01\) N.S. not significant.
Table 2.8 continued

(ii) Gastritic conditions

<table>
<thead>
<tr>
<th>Patient (age)</th>
<th>Clinical Condition</th>
<th>Incorporation Rate</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>mmol x $10^{-12}$/mg glycoprotein/h</td>
<td>Antrum</td>
<td>Lesser Curve</td>
<td>Greater Curve</td>
</tr>
<tr>
<td>Male, 72</td>
<td>Severe gastritis</td>
<td>142</td>
<td>202</td>
<td>142</td>
<td></td>
</tr>
<tr>
<td>Male, 56</td>
<td>Atrophic gastritis</td>
<td>-</td>
<td>112</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Male, 48</td>
<td>Gastritis, partial gastrectomy</td>
<td>-</td>
<td>205</td>
<td>249</td>
<td></td>
</tr>
<tr>
<td>Female, 78</td>
<td>Senile gastritis</td>
<td>126</td>
<td>109</td>
<td>152</td>
<td></td>
</tr>
<tr>
<td>Male, 18</td>
<td>Severe gastritis</td>
<td>157</td>
<td>183</td>
<td>94</td>
<td></td>
</tr>
<tr>
<td>Female, 82</td>
<td>Senile gastritis</td>
<td>139</td>
<td>161</td>
<td>104</td>
<td></td>
</tr>
<tr>
<td>Male, 38</td>
<td>Antral gastritis</td>
<td>118</td>
<td>153</td>
<td>167</td>
<td></td>
</tr>
</tbody>
</table>

Means ± S.E.M.  
- 136 ± 6.7**
- 154 ± 15.5NS
- 153 ± 27.6NS

Normal means ± S.E.M. (Table 2.7)  
- 234 ± 9.8
- 148 ± 9.6
- 151 ± 9.5

Students 't' test  
Gastritic antrum v normal**P < 0.01  
N.S. not significant.
### Table 2.8 continued

(iii) Other conditions

<table>
<thead>
<tr>
<th>Patient (age)</th>
<th>Clinical Condition</th>
<th>Incorporation Rate</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>mmol x 10^-12/mg glycoprotein/h</td>
<td>Antrum</td>
<td>Lesser Curve</td>
</tr>
<tr>
<td>Female, 85</td>
<td>Bleeding pre-pyloric ulcer</td>
<td>271</td>
<td>189</td>
<td>89</td>
</tr>
<tr>
<td>Female, 35</td>
<td>Multiple aphthous ulcers above pylorus</td>
<td>89</td>
<td>129</td>
<td>126</td>
</tr>
<tr>
<td>Female, 88</td>
<td>Ulcer on antrum</td>
<td>127</td>
<td>164</td>
<td>154</td>
</tr>
<tr>
<td>Male, 67</td>
<td>Carcinoma on lesser curve</td>
<td>208</td>
<td>95</td>
<td>175</td>
</tr>
<tr>
<td>Male, 57</td>
<td>Carcinoma on lesser curve</td>
<td>-</td>
<td>92</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>(60 near cancer)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male, 64</td>
<td>Carcinoma on greater curve</td>
<td>203</td>
<td>147</td>
<td>102</td>
</tr>
<tr>
<td>Normal means + S.E.M. (Table 2.7)</td>
<td>234 ± 9.8</td>
<td>148 ± 9.6</td>
<td>151 ± 9.5</td>
<td></td>
</tr>
</tbody>
</table>
Table 2.9  
Incorporation of N-acetyl-[\textsuperscript{3}H\textsubscript{]}glucosamine into an Acid Precipitable Fraction of Human Gastric Biopsy Samples from Patients Treated with Carbenoxolone

<table>
<thead>
<tr>
<th>Patient (age)</th>
<th>Clinical Condition</th>
<th>Carbenoxolone Treatment</th>
<th>Incorporation Rate mmol x 10\textsuperscript{-12}/mg glycoprotein/h</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Antrum</td>
</tr>
<tr>
<td>Male, 78</td>
<td>Ulcer on lesser curve</td>
<td>4 weeks</td>
<td>320</td>
</tr>
<tr>
<td>Female, 55</td>
<td>Ulcer on antrum</td>
<td>4 weeks</td>
<td>539</td>
</tr>
<tr>
<td>Female, 54</td>
<td>Ulcer on lesser curve</td>
<td>6 weeks</td>
<td>306</td>
</tr>
<tr>
<td>Female, 71</td>
<td>Ulcer on lesser curve</td>
<td>4 weeks</td>
<td>-</td>
</tr>
<tr>
<td>Male, 32</td>
<td>Ulcer on lesser curve</td>
<td>6 weeks (stopped 2 weeks previously)</td>
<td>270</td>
</tr>
<tr>
<td>Female, 44</td>
<td>Ulcer on lesser curve</td>
<td>6 weeks (ulcer not completely healed)</td>
<td>342</td>
</tr>
<tr>
<td>Male, 86</td>
<td>Antral gastritis and pre-pyloric ulcer</td>
<td>4 weeks</td>
<td>-</td>
</tr>
<tr>
<td>Female, 64</td>
<td>Gastric ulcer</td>
<td>6 weeks</td>
<td>-</td>
</tr>
<tr>
<td>Male, 65</td>
<td>Ulcer on antrum</td>
<td>6 weeks</td>
<td>-</td>
</tr>
<tr>
<td>Normal means ± S.E.M.</td>
<td></td>
<td></td>
<td>234 + 9.8</td>
</tr>
<tr>
<td>Subject</td>
<td>Clinical Condition</td>
<td>Carbenoxolone treatment</td>
<td>Incorporation Rate mmol x 10^{-12}/mg glycoprotein/h</td>
</tr>
<tr>
<td>-------------</td>
<td>-------------------------------</td>
<td>-------------------------</td>
<td>-----------------------------------------------</td>
</tr>
<tr>
<td>Female, 61</td>
<td>Ulcer on lesser curve</td>
<td>4 weeks</td>
<td>Antrum 207, 263</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>L. curve 58, 232</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>G. curve 129, 173</td>
</tr>
<tr>
<td>Male, 73</td>
<td>Antral gastritis, pre-pyloric ulcer</td>
<td>4 weeks</td>
<td>L. curve 67, 250</td>
</tr>
<tr>
<td>Male,</td>
<td>Irregularities of oesophagus and pylorus</td>
<td>4 weeks</td>
<td>Antrum 198, 549</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>L. curve 127, 262</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>G. curve 140, 299</td>
</tr>
<tr>
<td>Female</td>
<td>Pyloric ulcer</td>
<td>4 weeks</td>
<td>Antrum 277, 268</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>L. curve 94, 268</td>
</tr>
</tbody>
</table>

Normal values (Table 2.7): 234
### Table 2.11

**Incorporation of Sodium \(^{35}\text{S}\) Sulphate into an Acid Precipitable Fraction of Human Gastric Biopsy Samples**

<table>
<thead>
<tr>
<th>Subject</th>
<th>Clinical Condition</th>
<th>Incorporation Rate (mmol x 10(^{-12})/mg glycoprotein/h)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Male, 35</td>
<td>Apparent normal</td>
<td>Antrum: 1570, Lesser curve: 1750, Greater curve: 870</td>
<td></td>
</tr>
<tr>
<td>Female, 27</td>
<td>Apparent normal</td>
<td>1390, 1610, 1100</td>
<td></td>
</tr>
<tr>
<td>Male, 73</td>
<td>Partial gastrectomy</td>
<td>1550, 770</td>
<td></td>
</tr>
<tr>
<td>Female, 54</td>
<td>Ulcer on lesser curve</td>
<td>1250, 2350, 990</td>
<td></td>
</tr>
<tr>
<td>Female, 85</td>
<td>Gastric carcinoma on greater curve</td>
<td>380, 1440, 1890</td>
<td></td>
</tr>
</tbody>
</table>
Similarly a reduction in the uptake of hexosamine into glycoproteins from antral mucosal samples was observed in patients suffering from gastritic conditions while lesser and greater curve samples from the same patients were approximately normal [Table 2.8 (ii)].

In other pathological conditions of the stomach the same pattern of decreased incorporation of N-acetylglucosamine was observed in the diseased portion of the stomach. The patient with multiple aphthous ulcers above the pylorus had a low rate of uptake into the antral mucosa and the two patients with carcinoma of the lesser curve had reduced levels of uptake into biopsy samples from this area [Table 2.8 (iii)].

Carbenoxolone treatment was shown to reverse the trend of diminished incorporation associated with gastric ulceration (Table 2.9). In all cases carbenoxolone treatment resulted in healing the ulcer and an elevation in the uptake of N-acetylglucosamine. This effect was confirmed in the cases where biopsy samples were obtained on diagnosis of the ulcer and after treatment with the drug (Table 2.10).

The experiments on the incorporation of sodium sulphate into the acid precipitable fraction of human gastric biopsy samples showed that in all five subjects uptake of sulphate into the precipitated fraction did occur (Table 2.11). An elevation in the rate of uptake was seen in the lesser curve of a patient with an ulcerated lesser curve and in a cancerous greater curve sample but the significance of these results could not be tested because of the small number of patients studied.
2.4 DISCUSSION

Using the techniques of Shillingford (1975) it has been demonstrated that glycoprotein synthesis can be studied in discs of gastric tissue over incubation periods of at least four hours. The initial lag phase observed in these preliminary experiments possibly represents the time required for diffusion and activation of N-acetylglucosamine prior to its incorporation into high molecular weight molecules.

It is worth noting that cycloheximide at a concentration of $10^{-4}$M which has been shown to completely inhibit protein synthesis in the gut (Lukie and Forstner, 1972) also inhibits the incorporation of N-acetylglucosamine, confirming the glycoprotein synthesising ability of the tissue preparation used in these experiments. This inhibition, however is not complete, the incorporation of hexosamine into cycloheximide treated discs being approximately 60% of the control value after four hours incubation. This continued incorporation in the absence of protein synthesis may represent the addition of sugar units to incomplete oligosaccharide chains during intracellular transport and secretion.

Salivary and ovarian cyst mucus apparently possess a significant number of incomplete side chains (Carlson 1966; Lloyd et al., 1968), and similarly, incomplete side chains in stored gastric mucus could provide a large reservoir of endogenous acceptor for N-acetylglucosamine incorporation.

Subsequent experiments showed the incorporation assay to be a useful tool for the in vitro measurement of the in vivo effects of drugs on glycoprotein synthesis. A similar increase in the rate of incorporation to that seen in experiments in which N-acetyl$[^3]$H$[^3]$glucosamine was administered in vivo after carbenoxolone treatment (Shillingford, 1975)
was observed using the *in vitro* incorporation system. The *in vitro* assay, however, has the advantage of being more economical in the use of radiolabelled substrates and does not suffer from the disadvantage of distribution of the labelled compound to other tissues and subsequent contamination of the isolated gastric fractions with radiolabelled secretory products from these tissues e.g. salivary mucus and blood.

The stimulation by carbenoxolone of the incorporation of N-acetylglucosamine into rat and N-acetylglucosamine, glucosamine, N-acetylneuraminic acid and fucose into ferret gastric glycoproteins is in agreement with the work of other authors who found elevated levels of hexosamines (Shillingford et al., 1973) and N-acetylneuraminic acid (Gheorghiu et al., 1972; Shillingford et al., 1973) in rat gastric glycoproteins by chemical analysis after carbenoxolone treatment.

The increased incorporation of sugars without a concomittant increase in the incorporation rate of amino acids indicates that the primary effect of carbenoxolone is to modify the carbohydrate side chain of the glycoprotein rather than cause an overall increase in the rate of glycoprotein synthesis or an alteration in the polypeptide backbone. However, the non-significant trend towards increased incorporation of serine and threonine suggests that an effect on protein synthesis cannot be ruled out completely.

The absence of increased incorporation of galactose into ferret gastric mucosal glycoproteins after carbenoxolone treatment is difficult to interpret in view of the increase seen with the other sugars, particularly if, as the case in human gastric mucin, a disaccharide of N-acetylglucosamine and galactose constitutes a major portion of the
carbohydrate side chain (Oates et al., 1974).

However, carbenoxolone has also been shown to have anomalous effects on galactose incorporation in the rat stomach. Shillingford et al. (1973) found that pretreatment with the drug caused a decrease in the incorporation rate of galactose. It is possible that carbenoxolone as well as producing a more heavily glycosylated glycoprotein, as evidenced by the increased uptake of N-acetylglucosamine, fucose and N-acetylneuraminic acid, may also result in the production of a modified carbohydrate side chain characterised by a low galactose content.

A possible explanation of the action of carbenoxolone on the incorporation of the component sugars of the carbohydrate chain is that the activity or specificity of glycosyltransferases which assemble the oligosaccharide is altered. In particular an action on the transferases responsible for the addition of the first few sugars would effect the specificity and activity of all subsequent glycosyltransferases and hence the structure of the oligosaccharide produced.

It has previously been shown that factors associated with mucosal damage such as restraint (Lambert et al., 1969) and aspirin administration (Rainsford, 1975) led to a decrease in the sulphate content in rat gastric mucus. Moreover, this decrease has been shown to be the result of a decrease in synthesis rather than an increase in the catabolism of sulphated glycoproteins (Dziewiatkowski, 1964). Carbenoxolone was shown to have the opposite effect causing an increase in the extent of sulphation of the glycoproteins. This effect, at least in part, may explain the anti-peptic action of carbenoxolone (Birnbaum and Karmeli, 1975) since sulphated poly-anions have been shown to inhibit pepsin (Martin et al., 1968).

The drug-induced increase in the incorporation of sulphate was apparent after both acid and cetylpyridinium chloride precipitation. The level of
incorporation into these materials precipitated by the two methods was however markedly different, that in the cetylpyridinium chloride precipitate being several orders of magnitude greater than the acid precipitate. The reason for this difference is in the nature of the fraction isolated. Acid, being a non-specific precipitating agent, results in the isolation of a fraction containing macromolecules from non-mucosal and mucosal cells containing neutral glycoproteins as well as sulphated glycoproteins. The weight of the precipitate was 50-60 mg. Sulphate incorporation when related to this weight is low. In contrast cetylpyridinium chloride is relatively specific, resulting in the precipitation of acid macromolecules such as nucleic acids and sulphated glycoproteins (weight of precipitate 3-5 mg), due to the formation of quaternary ammonium complexes (Scott, 1960). Hence considerably higher incorporation rates are seen using this method.

Although conclusive evidence for the involvement of alcohol in human gastric ulceration is lacking (Cooke, 1976) a number of authors have shown it to cause mucosal damage in animals (Williams, 1969; Eastwood and Kirchner, 1974). These reports are supported by the dramatic decrease in the incorporation of N-acetylglucosamine and the development of gastric erosions after ethanol administration.

Davenport (1967) found that gastric mucosal damage, measured by a drop in the transmucosal potential due to back diffusion of hydrogen ions occurred after ethanol administration. Although this back diffusion may stem from a general cytotoxic effect on the mucosal cells it may also reflect a direct effect of alcohol on mucus synthesis causing a weakened mucosal barrier.

Mucus synthesis, measured by the rate of incorporation of N-acetylglucosamine was shown to vary between the anatomical divisions of the human
stomach. This reflects the distribution of mucus secreting cells throughout the stomach, the antrum having the highest concentration. The results do, however, support the protective role of mucus in the stomach since the antrum is a low risk area with regard to ulceration, whereas the lesser curve may be regarded as a high risk area (Johnson, 1965).

Disease states of the stomach such as gastric ulcer, gastritis and carcinoma have been shown to be associated with a decrease in the incorporation of N-acetylglucosamine, indicating a malfunction in mucous glycoprotein synthesis. This conclusion is supported by the findings of Domschke et al (1972) that N-acetylneuraminic acid levels were depleted in mucus from gastric ulcer patients.

The production of sulphated glycoproteins was also shown to be altered in disease states of the stomach. In contrast to the decreased sulphation seen in animals (Lambert and Andre, 1969) human gastric lesions are associated with increased sulphation of glycoproteins. This finding is in agreement with the postulate of Lambert and Andre (1972) that human gastric disease is associated with an increased secretion of sulphated mucin.

The same authors suggest that normal human gastric mucosa does not secrete appreciable amounts of sulphated mucin and that the presence of sulphated glycoproteins in gastric aspirates (Schrager and Oates, 1971) is due to contamination by salivary and bronchial secretions. The occurrence of sulphate incorporation into endoscopically diagnosed normal mucosae does not support this hypothesis. Further indications of the ability of the human gastric mucosa to produce sulphated glycoproteins is provided by the demonstration of their presence in extracts from surgically removed gastric tissue (Schrager and Oates, 1974).
The trend towards decreased incorporation of N-acetylglucosamine in biopsy samples from gastritic and gastric ulcer patients could be reversed by carbenoxolone therapy. The results suggest that carbenoxolone may have the same effect in man as in rats and ferrets, perhaps resulting in the production of a mucus glycoprotein with an altered carbohydrate side chain and an increased carbohydrate to protein ratio, having greater resistance to proteolytic attack (Menguy and Desbaillets, 1968) and thus being able to afford protection to the mucosa.

Similarly, it may be argued that gastric ulcer is associated with the production of an under-glycosylated mucin or with a modified oligosaccharide chain structure within the mucin. Evidence exists that disease states of the stomach, such as carcinoma, may be associated with changes in the quantitative relationships of the sugars comprising the carbohydrate portion of the mucin. (Schrager and Oates, 1973). Such a modified glycoprotein may not have the protective ability of normal mucus glycoprotein. A depletion in N-acetyleneuraminic acid, suggested as being essential for the protective viscous properties of mucus (Gottschalk, 1960) has been shown in gastric ulcer patients (Domschke, 1972).

It may be postulated that the primary cause of gastric ulceration is a derangement in glycoprotein synthesis leading to impaired mucosal defence. Such a derangement could occur at the polysomal level as is believed to be the case with the carcinogenic lesion (Webb et al., 1965) which appears to be related to ulceration in the stomach with regard to incorporation of hexosamines. It is worth noting that gastric ulcers, particularly on the greater curve have a tendency to become malignant and gastric carcinomas frequently ulcerate (Montgomery and Richardson, 1975).
CHAPTER THREE

THE EFFECTS OF DRUGS ON GLYCOPROTEIN SYNTHESIS
IN RAT GASTRIC MUCOSAL SCRAPINGS
3.1. INTRODUCTION

The work of the previous chapter showed that glycoprotein synthesis and the effects of ulcerogenic and antiulcerogenic drugs upon this synthesis could be studied in discs of tissue taken from the stomachs of animals.

This chapter contains details of an alternative system for studying glycoprotein synthesis in the stomach. The technique is based on a further modification of the method of Lukie and Forstner (1972) and measures the incorporation of radiolabelled precursors into an acid precipitable fraction of rat gastric mucosal scrapings.

It was felt that the use of mucosal scrapings would give a more accurate reflection of glycoprotein synthesis since the fraction isolated after incubation would contain macromolecules of mucosal origin without contamination by components of the muscular and connective tissue layers of the stomach. Exclusion of these contaminants should lead to increased incorporation rates with the use of homogenised mucosal scrapings.

Using this technique the effect of a number of anti-ulcerogenic and ulcerogenic drugs on glycoprotein synthesis was studied both when added to an isolated mucosal cell homogenate and when administered to animals before determination of the in vitro incorporation of hexosamine.

The anti-ulcerogenic drugs used in these studies were carbenoxolone which has been discussed in the previous chapter, a carbenoxolone analogue BX363A, the E type prostaglandins and their synthetic derivatives (see Figs. 1.4 and 3.1) and L-glutamine. The ulcerogenic drugs studied were indomethacin, ethanol and penicillin G.

A great number of reports have appeared on the anti-ulcer effect of the prostaglandins and prostaglandin analogues. Most of the work in this
Figure 3.1  Structures of BX363A and Prostaglandin Analogues

- BX 363A
- 16, 16 dimethyl PGE₂
- 15,15 methyl PGE₂ methyl ester
- AY 22469
field, however, has been concentrated on trying to prove a relationship between the anti-ulcer effect of these compounds and their ability to inhibit gastric acid secretion (Bass, 1974), possibly via cyclic AMP (Bieck et al., 1973). Few authors have considered the effect of prostaglandins on mucus synthesis and secretion in relation to ulcer healing. Fung et al. (1974a) have demonstrated endoscopically and histologically that the prostaglandin analogue 15-R-15 methyl PGE2 methyl ester, which has been shown to be beneficial in the treatment of gastric ulceration (Fung et al., 1974b), has a powerful stimulatory effect on the production of gastric mucus in ulcer patients. Thus it is possible that the other prostaglandins which have been shown to be antiulcerogenic in animals, PGE2 (Lippmann, 1974), PGE1 (Robert et al., 1968), compound AY22469 (Lippmann, 1974) and 16,16 dimethyl PGE2 (Robert et al., 1976) may also have an effect on mucus synthesis and exert their beneficial action through such an effect.

Similarly, the amino acid 1-glutamine has been shown to be effective in suppressing gastric ulceration induced by stress in rats (Takagi and Okabe, 1968) and aspirin (Okabe et al., 1974; Tanaka et al., 1974). These authors have related this beneficial effect to the ability of 1-glutamine to inhibit the back diffusion of gastric HCl although they do not postulate a mechanism for this inhibition. Again an effect on mucus production has not been considered.

Despite the lack of a positive correlation between indomethacin use and chronic gastric ulceration in humans (Cooke, 1976) the drug has been shown to be ulcerogenic in animals (Nicoloff, 1968) and to cause acute gastric mucosal lesions in patients with rheumatoid arthritis (Menguy, 1969). In animals the development of the lesion has been shown to be associated with a decrease in the concentration of protein bound carbohydrates, particularly hexosamines and sialic acids, of gastric mucus. Thus, the
ulcerogenic effect of the drug may in part be mediated through an
effect on glycoprotein synthesis.

Similarly, ethanol (Williams, 1969) and penicillin G (Lyle, 1974)
have been shown to be associated with mucosal lesions in animals and
humans. In the case of penicillin G the active mucosal damaging agent
is thought to be penicillamine, a product of penicillin G metabolism.
3.2 MATERIALS AND METHODS

Chemicals

Carbenoxolone sodium and compound BX363A were gifts from Biorex Laboratories Ltd., London. Prostaglandins E₁, E₂, 15-R-15 methyl prostaglandin E₂ methyl ester and 16,16 dimethyl prostaglandin E₂ were gifts from Dr. J.E. Pike, The Upjohn Company, Kalamazoo, Michigan. The prostaglandin analogue AY 22469 was donated by Dr. W. Lippman, Ayerst Laboratories, Quebec. Radiochemicals (Table 2.2) were purchased from The Radiochemical Centre, Amersham. Sephadex G-200 and ConA-Sepharose were obtained from Pharmacia (G.B.) Ltd., London.

Animals

The animals used in this series of experiments were female Wistar albino rats (200-250g).

Incorporation of N-acetyl-[³H]glucosamine into an Acid Precipitable Fraction of Rat Gastric Mucosal Scrapings

Rats were killed by cervical dislocation and the stomachs isolated and washed as previously described (Chapter 2.2). The stomachs were stretched out on a glass plate and the layer of mucus lining the glandular area of the stomach removed by lightly scraping with a glass microscope slide. The mucosa was then separated from the muscular layer, again by scraping with a glass slide. The mucosal scrapings from each stomach were homogenised in 15 ml incubation medium using a Potter-Elvejehøm homogeniser. The medium was that of Lukie and Forstner (1972) used previously (NaCl, 119 mM; KCL, 5.6 mM; CaCl₂ 3.0 mM; KH₂PO₄, 1.4 mM; NaHCO₃, 29.3 mM; L-glutamine, 80 mM).
Aliquots (5 ml) of homogenised mucosal scrapings were incubated in a Mickle shaking incubator at 37°C and 100 oscillations/min. Incubation periods were for 4 hours, except where specified, and were started (after a preincubation period of 10 min) by the addition of N-acetyl-[3H]glucosamine (2.5μCi) in a small volume of 0.9% NaCl (50μl). Throughout the incubation flasks were flushed with O2-CO2 (95%-5% v/v) every 10 min. Incorporation was terminated by the addition of 5 ml trichloroacetic acid:tungstophosphoric acid (20% w/v: 2% w/v).

The fraction precipitated overnight was collected by centrifugation at 2,000 g for 10 min in an MSE Mistral centrifuge. The material was washed twice with distilled water, extracted twice for lipids with chloroform-methanol (1:1), air dried and weighed.

Studies on the relative rates of incorporation of other sugars were performed by adding amounts equivalent to 2.5μCi of N-acetyl[3H]glucosamine (6.25 x 10^{-10} mol). In further studies on the relative incorporation rates of different sugars the assays were carried out using 50x10^{-5}M concentrations of the sugar being investigated.

**Determination of Radioactivity**

Radioactivity incorporated into the precipitated fraction was determined by the method used in the previous chapter. In this case solubilisation was with perchloric acid (0.2 ml, 60% w/v) and hydrogen peroxide (0.4 ml, 100 vols). Scintillation counting was carried out in a Packard Tri-Carb Spectrometer (Model 3320) using an external standard channels ratio method for determination of counting efficiencies or in an LKB Wallac Ultrabeta 2002 scintillation counter, programmed for d.p.m. calculations based on internal standard channels ratio efficiency determinations.
Examination of Drug Effects In Vivo and In Vitro

To examine the in vitro effects of drugs on the incorporation assay they were added in a small volume of vehicle to the flasks containing the mucosal preparation prior to incubation. Control flasks received vehicle only. In these experiments aliquots from a bulked mucosal homogenate, prepared from a number of stomachs, were used.

The in vivo effects of drugs were determined by pretreatment of the animals prior to killing. Control animals were treated identically but with the omission of the drug. Details of the drugs and dosing regimes used are given in the Experimental and Results section.

Analysis of Radioactively Labelled Rat Gastric Mucosal Glycoprotein by Concanavalin A-Sepharose Affinity and Sephadex G-200 Gel Chromatography.

Column preparation

Sephadex G-200 (superfine) was equilibrated in sodium bicarbonate (0.1M) for 3 days at room temperature and packed into a chromatography column (40 x 3 cm) using the technique outlined by the manufacturers (Sephadex, Gel Filtration in Theory and Practice). The void volume was determined using Blue Dextran 2000 and the column calibrated for molecular weight determinations by measuring the volume of elution of cytochrome C (M.W. 13,000), ovalbumin (M.W. 45,000), bovine serum albumin (M.W. 67,000) and yeast alcohol dehydrogenase (M.W. 150,000). A calibration curve was plotted using the relationship:

\[
\frac{v_e}{v_o} = k \log M
\]

in which, \(v_e\) = volume of elution, \(v_o\) = void volume, \(M\) = molecular weight and \(k\) is a constant.
Concanavalin A-Sepharose was equilibrated with sodium bicarbonate (0.1M) for 3 days at room temperature and packed into a chromatography column (20 x 0.9 cm). On settling the column was washed with further additions of sodium bicarbonate.

Sample preparation

Incorporation of N-acetyl[\(^3\)H]glucosamine into a bulked mucosal homogenate from 6 rats was carried out as previously described. Incubations were terminated by the addition of ice cold distilled water and the combined contents of the flasks exhaustively dialysed against distilled water (5 changes) at 4°C for 24 hr to remove free N-acetylglucosamine and radiolabelled low molecular weight molecules. After centrifugation to remove water insoluble materials the supernatant of the dialysate, containing water soluble glycoproteins, was freeze-dried. The lyophilized material was redissolved in 10 ml NaHCO\(_3\) (0.1M) and applied to the Concanavalin A-Sepharose column.

Chromatographic separation

Material was eluted from the Concanavalin A-Sepharose column using the following eluants:

1) sodium bicarbonate (0.1M)
2) sodium chloride (1M)
3) distilled water
4) methyl-\(\alpha\)-D-glucopyranoside (50 mg/ml).

Methyl-\(\alpha\)-D-glucopyranoside which has a high affinity for Concanavalin A was used to displace glycoproteins from the column. Fractions (3 ml) eluted with this material were analysed for radioactivity. Those containing radiolabelled material were combined (final volume, 36 ml) dialysed against distilled water to remove the \(\alpha\)-methylglucose and lyophilised.
The dried material was redissolved in 5 ml NaHCO₃ (0.1M) and applied to the Sephadex G-200 column. The column was eluted with NaHCO₃, flow rate 0.10 ml/h, operating pressure 15 cm H₂O, and 3 ml fractions collected. Aliquots (0.5 ml) of each fraction were analysed for radioactivity and the protein content of each fraction measured by the method of Lowry et al (1951).

An analysis of the glycoprotein from rats pretreated with carbenoxolone (25 mg/kg/day, for 7 days) was carried out using the same procedures.

**Hydrolysis of Radioactively Labelled Rat Gastric Mucosal Glycoprotein**

The fractions from the Sephadex column described above which were shown to contain radiolabelled glycoprotein were combined and freeze dried. The material was used in the following hydrolysis studies.

**Sialic acids**

Sialic acid residues were cleaved from the glycoprotein (10 mg freeze dried material) by hydrolysis under nitrogen in a sealed container for 40 min with 0.1 N H₂SO₄ (2 ml) at 80°C (Gottschalk, 1960). After cooling, the hydrolysis mixture was made neutral by addition of saturated Ba(OH)₂ and mixed with ethanol (3 vol). After 1h at 4°C, the precipitated mucin and BaSO₄ were deposited by centrifugation and the supernatant was shaken with Dowex 50 (H⁺ form) resin (1 vol resin:5 vol of supernatant). The cation-free solution was decanted and the resin was washed once with water (5 vol). The combined supernatant and washings were freeze dried and the residue containing sialic acid dissolved in water (200μl) and examined by thin layer chromatography.
Neutral sugars

Neutral sugars were released from the glycoprotein material (10 mg) by hydrolysis under nitrogen in 0.5N H₂SO₄ (2 ml) for 3h at 105°C (Mah, 1961). The resulting hydrolysate was passed down a column (1 cm x 10 cm) of Amberlite Monobed 3 to remove acid residues. The column eluate (about 50 ml) was freeze dried and redissolved in water (200μl) and examined by thin layer chromatography.

Amino sugars

The glycoprotein fraction (10 mg) was hydrolysed under nitrogen in 2 NHCl (2 ml) at 105°C for 16h. (Blix, 1948). The resulting humin was centrifuged and the hydrolysate was evaporated to dryness in vacuo at room temperature over P₂O₅ and NaOH. The dried residue was dissolved in water (200μl) and examined by thin layer chromatography.

Thin Layer Chromatographic separations

The products of the preceding hydrolyses were examined by thin layer chromatography. The adsorbant and solvent systems used are shown in Table 3.1. Duplicate samples (50μl hydrolysate) were spotted on the chromatography plate. Relevant standards of cold sugars (0.5 mg) were run in parallel. After running sugars were detected on the plates with alkaline silver nitrate. Coincidence of radioactivity with the sugar spots was determined by scanning the T.L.C. plates using a Berthold LB 27723 radioplate scanner.
<table>
<thead>
<tr>
<th>Sugar</th>
<th>Adsorbant</th>
<th>Solvent</th>
<th>System</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neutral sugars</td>
<td>Silica gel G + 0.02M sodium acetate</td>
<td>Acetone:water (9:1)</td>
<td>I</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Cellulose</td>
<td>Pyridine: ethylacetate: acetic acid: water (5:1:3:1)</td>
<td>II</td>
</tr>
<tr>
<td>Amino sugars</td>
<td>Cellulose</td>
<td>Pyridine: ethylacetate: acetic acid: water (5:1:3:1)</td>
<td>II</td>
</tr>
<tr>
<td></td>
<td>Silica gel</td>
<td>N-propanol: ethylacetate: water:15 M NH₃ (5:1:3:1)</td>
<td>III</td>
</tr>
<tr>
<td>Sialic acids</td>
<td>Cellulose</td>
<td>Ethanol: water: ammonia soln. (sp.gr.0.88) (80:20:1)</td>
<td>IV</td>
</tr>
</tbody>
</table>
3.2 EXPERIMENTAL AND RESULTS

The uptake of N-acetyl-[3H]glucosamine into the acid precipitable glycoprotein fraction of rat gastric mucosal scrapings was shown to be approximately linear for at least four hours (Fig. 3.2). This incorporation was shown to be dependent on the presence of oxygen (Fig. 3.2) and absent when the mucosal preparation was denatured by heating (Fig. 3.3).

The assay was shown to have similar characteristics to that used in Chapter 2 in that the level of incorporation could be reduced to approximately 60% of the control value by the presence of the protein synthesis inhibitor cycloheximide at a concentration of 10^{-4} M (Fig. 3.4). Also the optimal concentration for the substrate in the modified Krebs medium of 8 mM glutamine (Fig. 3.5) is similar to that required for incorporation into gastric slices (Shillingford, 1975).

Antibiotics were not added routinely to the incubation medium to inhibit the effects of contaminating bacteria since the antibiotic cycloheximide at a concentration of 1.5 \mu g/ml showed no effect on the uptake of hexosamine over the four hour period (Fig. 3.6). In an attempt to determine whether the stomach microflora were capable of incorporating significant amounts of N-acetylglucosamine into acid precipitable glycoproteins the washed stomachs, prior to incubation, were agitated with medium for 5 min. to release adherent microorganisms and the medium alone incubated with N-acetyl-[3H]glucosamine for 4 hours. Total incorporation of radioactivity into acid precipitable material was less than 1% of the incorporation occurring with mucosal scrapings.
Fig. 3.2 Effect of oxygen on the incorporation of N-acetyl-[3H]-glucosamine into gastric mucosal scrapings

Results are plotted as means ± SEM of three observations. Open triangles represent unoxygenated mucosa, closed triangles oxygenated mucosal scrapings.
Fig. 3.3 Incorporation of N-acetyl-[\textsuperscript{3}H]-glucosamine into control and denatured mucosal scrapings.

![Graph showing incorporation rate over time with error bars](image1)

Results are plotted as means ± SEM of four observations. Closed triangles represent control incubation, open triangles represent incubation with denatured mucosal scrapings.

Fig. 3.4 Effect of 10\textsuperscript{-4}M cycloheximide on the incorporation of N-acetyl-[\textsuperscript{3}H]-glucosamine into rat gastric mucosal scrapings.

![Graph showing incorporation rate with error bars](image2)

Results are plotted as means ± SEM of three observations. Closed triangles represent control incubation, open triangles represent incubation in the presence of cycloheximide (10\textsuperscript{-4}M).
Fig. 3.5 The effect of glutamine concentration on the incorporation of N-acetyl-[\textsuperscript{3}H]-glucosamine into glycoproteins from gastric mucosal scrapings. Results are plotted as means ± SEM of four observations.
on the incorporation of N-acetyl-[³H]glucosamine into acid precipitable glycoproteins of rat gastric mucosal scrapings

Results are plotted as means ± SEM of three observations. Closed triangles represent control incorporation. Open triangles incorporation in the presence of cycloheximide.
The In Vitro Effects of Compounds on the Incorporation of N-acetyl-[\textsuperscript{3}H] glucosamine

The in vitro effects of carbenoxolone, indomethacin, ethanol, and penicillin G on the uptake of hexosamine were determined by the addition of the compounds in small volumes (50\,\mu l, except for ethanol, 500\,\mu l) of aqueous solutions or suspensions (indomethacin) to the mucosal preparations prior to the addition of N-acetyl-[\textsuperscript{3}H]glucosamine. The final concentration of the drugs and the results of the incorporation assays are shown on Table 3.2.

A series of natural, synthetic and semi-synthetic prostaglandins, consisting of PGE\textsubscript{1}, PGE\textsubscript{2}, 15-R-15 methyl PGE\textsubscript{2} methyl ester, 16,16 dimethyl PGE\textsubscript{2} and compound AY22469, and cyclic AMP were also tested for their in vitro effects on the incorporation of N-acetyl-[\textsuperscript{3}H]-glucosamine. Cyclic AMP was added to the mucosal preparation as an aqueous solution (50\,\mu l) to give a final concentration range of 10\textsuperscript{-12}-10\textsuperscript{-4}\,M. The prostaglandins were added in 50\,\mu l of 3\% ethanol to give a final concentration range of 6-180\,\mu M. Control preparations were treated with the respective solvents. The results of the incorporation studies are shown in Table 3.3.

Of the compounds added to the incubation medium only the prostaglandin analogue AY22469 appeared to increase the incorporation of the amino sugar. This increase however was slight (approx. 12\%) and did not appear to be concentration-dependent over the range of concentrations used. The other prostaglandins studied in vitro either had no effect on the incorporation (15-R-15 methyl PGE\textsubscript{2} methyl ester and 16,16 dimethyl PGE\textsubscript{2}) or showed a
Table 3.2 Effects of ethanol, carbenoxolone, indomethacin and penicillin G in vitro on the rate of incorporation of N-acetyl-[\(^3\)H]-glucosamine into the acid-precipitable fraction of rat gastric mucosal scrapings

<table>
<thead>
<tr>
<th>Compound added</th>
<th>Final concn. in incubation medium</th>
<th>Incorporation rate n mol x 10(^{-4})/mg glycoprotein</th>
<th>Change from control %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>Experimental</td>
<td>Control</td>
<td>Experimental</td>
</tr>
<tr>
<td>Carbenoxolone</td>
<td>10(\mu)M</td>
<td>69.61±6.6 (4)</td>
<td>65.11, 67.86</td>
</tr>
<tr>
<td></td>
<td>100(\mu)M</td>
<td>69.61±6.6 (4)</td>
<td>62.28, 65.64</td>
</tr>
<tr>
<td></td>
<td>1mM</td>
<td>69.61±6.6 (4)</td>
<td>50.76, 44.18</td>
</tr>
<tr>
<td>Indomethacin</td>
<td>10(\mu)M</td>
<td>69.45±2.45 (4)</td>
<td>69.0, 66.86</td>
</tr>
<tr>
<td></td>
<td>100(\mu)M</td>
<td>69.45±2.45 (4)</td>
<td>66.34, 68.79</td>
</tr>
<tr>
<td></td>
<td>1mM</td>
<td>69.45±2.45 (4)</td>
<td>52.87, 49.97</td>
</tr>
<tr>
<td>Ethanol</td>
<td>10% v/v</td>
<td>54.7±3.8 (6)</td>
<td>11.5 ± 0.75(6)</td>
</tr>
<tr>
<td>Penicillin G</td>
<td>200 units/ml</td>
<td>93.5±7.73(3)</td>
<td>28.5 ± 0.23(3)</td>
</tr>
</tbody>
</table>

Results are shown as means ± S.E.M. where the number of observations was greater than two (numbers in parentheses indicating number of observations).

Students' t-test *** p < 0.001
Table 3.3  Effects of PGE₁, PGE₂, 15-R-15 methyl PGE₂ methyl ester, 16,16 dimethyl PGE₂, AY 22469 and cyclic AMP in vitro on the rate of incorporation of N-acetyl-[³H]glucosamine into the acid-precipitable fraction of rat gastric mucosal scrapings

<table>
<thead>
<tr>
<th>Compound added</th>
<th>Final concn in incubation medium</th>
<th>Incorporation Rate nmol x 10⁻⁴/mg glycoprotein</th>
<th>Change from control %</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Experimental</td>
<td></td>
</tr>
<tr>
<td>PGE₁</td>
<td>6 - 180μM</td>
<td>47.79 ± 1.76(4)</td>
<td>43.62 ± 0.8 (8)</td>
</tr>
<tr>
<td>PGE₂</td>
<td>6 - 180μM</td>
<td>62.03 ± 1.37(4)</td>
<td>54.19 ± 1.1 (8)</td>
</tr>
<tr>
<td>15-R-15 methyl PGE₂</td>
<td>6 - 180μM</td>
<td>61.50 ± 0.46(4)</td>
<td>60.21 ± 0.74(8)</td>
</tr>
<tr>
<td>16,16 dimethyl PGE₂</td>
<td>6 - 180μM</td>
<td>63.48 ± 1.08(4)</td>
<td>61.70 ± 2.03(8)</td>
</tr>
<tr>
<td>AY 22469</td>
<td>6 - 180μM</td>
<td>54.85 ± 1.27(4)</td>
<td>61.53 ± 0.86(8)</td>
</tr>
<tr>
<td>Cyclic AMP</td>
<td>10⁻¹²-10⁻⁴ M</td>
<td>59.39 ± 2.94(3)</td>
<td>60.47 ± 1.24(9)</td>
</tr>
</tbody>
</table>

Prostaglandins were added in 50 μl aqueous ethanol to give final concentrations of 6, 12, 30, 60, 90, 120, 150 and 180μM.

Cyclic AMP was added in 50μl aqueous solution to give final concentrations of 10⁻¹², 10⁻¹¹ ....... 10⁻⁴M.

Results are shown as means ± S.E.M. where the number of observations was greater than two (numbers in parentheses indicating the number of observations).
slight inhibition ($\text{PGE}_1$ and $\text{PGE}_2$) of approximately 10%. Again these changes did not show a concentration-dependency.

Cyclic AMP was shown to have no effect in vitro on the uptake of hexosamine over the concentration range used.

All of the other compounds examined in vitro resulted in decreased incorporation of N-acetylglucosamine. Ethanol was by far the most potent inhibitor and at the concentration used caused a 78% decrease in the rate of incorporation. Carbenoxolone and indomethacin appeared to have a concentration dependent effect, 1mM concentrations showing inhibitions of 32% and 24% respectively. Similarly penicillin G caused an inhibition of 30% at a concentration of 200 units/ml.

The Effects of Pretreatment of Rats with Carbenoxolone on the Incorporation of N-acetylglucosamine, Glucosamine, Glucose, Galactose and Galactosamine into an Acid Precipitable Fraction of Rat Gastric Mucosa

Rats treated with carbenoxolone (25mg/kg/day for seven days) were sacrificed and mucosal preparations made. The incorporation of N-acetyl-$[^3\text{H}]$-glucosamine, $[^3\text{H}]$-glucosamine, $[^3\text{H}]$-glucose, $[^3\text{H}]$-galactose and $[^1\text{C}]$-galactosamine was carried out with and without the addition of unlabelled sugars (5 x $10^{-5}$M concentrations of each respective sugar). The results are shown in Table 3.4 and Table 3.5.

The experiments in which labelled sugar only was added to the incorporation assay (Table 3.4) showed that the sugars were not equally incorporated into the precipitable glycoprotein fraction. N-acetylglucosamine was by far the best substrate for the assay, followed, in order of descending incorporation rate, by glucosamine, galactose, glucose and galactosamine.
Table 3.4  Effects of carbenoxolone on the incorporation of $6.25 \times 10^{-10}$M concentrations of N-acetyl$[^3$H$]$glucosamine, $[^3$H$]$glucosamine, $[^3$H$]$glucose, $[^3$H$]$galactose and $[^1$4$C$]$galactosamine into an acid precipitable fraction of rat gastric mucosal scrapings.

<table>
<thead>
<tr>
<th>Sugar</th>
<th>Incorporation rate nmol x $10^{-5}$/mg glycoprotein/4h</th>
<th>Change from control (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Carbenoxolone treated</td>
</tr>
<tr>
<td>N-acetyl$[^3$H$]$ glucosamine</td>
<td>416 ± 83.8 (4)</td>
<td>754 ± 39.5 (4)</td>
</tr>
<tr>
<td>$[^3$H$]$glucosamine</td>
<td>153 ± 7.5 (4)</td>
<td>302 ± 19.1 (4)</td>
</tr>
<tr>
<td>$[^3$H$]$glucose</td>
<td>1.98 ± 0.33 (4)</td>
<td>3.92 ± 0.15 (4)</td>
</tr>
<tr>
<td>$[^3$H$]$galactose</td>
<td>3.7 ± 0.19 (4)</td>
<td>2.4 ± 0.29 (4)</td>
</tr>
<tr>
<td>$[^1$4$C$]$galactosamine</td>
<td>1.92 ± 0.26 (4)</td>
<td>3.53 ± 0.50 (4)</td>
</tr>
</tbody>
</table>

Results are expressed as means ± S.E.M. of the number of observations shown in parentheses.

Students' $t$-test  * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. 
Table 3.5 Effect of carbenoxolone on the incorporation of $5 \times 10^{-5}$ M concentrations of N-acetylglucosamine, glucosamine, glucose, galactose and galactosamine into an acid precipitable fraction of rat gastric mucosal scrapings.

<table>
<thead>
<tr>
<th>Sugar</th>
<th>Incorporation rate (nmol/mg glycoprotein/4h)</th>
<th>Change from control (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Carbenoxolone treated</td>
</tr>
<tr>
<td>N-acetylglucosamine</td>
<td>12.9 ± 0.52(4)</td>
<td>29.1 ± 1.72(4)</td>
</tr>
<tr>
<td>Glucosamine</td>
<td>11.7 ± 0.24(4)</td>
<td>29.2 ± 1.26(4)</td>
</tr>
<tr>
<td>Glucose</td>
<td>4.5 ± 0.23(4)</td>
<td>3.95 ± 0.07(4)</td>
</tr>
<tr>
<td>Galactose</td>
<td>6.8 ± 0.53(4)</td>
<td>7.3 ± 0.28 (4)</td>
</tr>
<tr>
<td>Galactosamine</td>
<td>3.2 ± 0.56(4)</td>
<td>5.7 ± 0.68 (4)</td>
</tr>
</tbody>
</table>

Results are expressed as means ± S.E.M. of the number of observations shown in parentheses.

Students' t-test * P < 0.05, *** P < 0.001, N.S. not significant.
Carbenoxolone pretreatment caused a significant increase in the rate of uptake of N-acetylglucosamine, glucosamine, glucose and galactosamine. In contrast, however, the rate of incorporation of galactose was significantly reduced (-35%) after carbenoxolone treatment.

Substantially different results were produced when the same incorporation assays were carried out in the presence of $5 \times 10^{-5}$M concentrations of the sugars (Table 3.5). Whereas N-acetylglucosamine and glucosamine were again incorporated at higher rates than the other sugars the uptake of glucosamine was equal to N-acetylglucosamine. The effect of carbenoxolone in producing a significant increase in the uptake of glucose and reduction in that of galactose was not apparent at this higher concentration of sugar.

The Effect of Pretreatment of Rats with Carbenoxolone, BX 363A, Penicillin G, Ethanol and Indomethacin on the Incorporation of N-acetyl-[3H]-glucosamine

The in vivo effects of these drugs was determined by pretreatment of rats prior to sacrifice. The dosing regimes used are shown on Table 3.6 and the results of the incorporation assays in Table 3.7.

As in the previous studies carbenoxolone was shown to cause an increase in the rate of uptake of hexosamine into the precipitable glycoprotein fraction. Seven days dosing with carbenoxolone showed an increase of 51% and fourteen days dosing and increase of 215% in the rate of incorporation over controls. An increase of 35% was observed after seven days dosing with BX 363A.

Penicillin G, after seven days dosing, and ethanol one hour after a single dose, caused inhibitions in the rate of incorporation
Table 3.6  Dosing regimes used to determine the effects of carbenoxolone BX 363A, penicillin G, ethanol and indomethacin on gastric glycoprotein synthesis

<table>
<thead>
<tr>
<th>Compound</th>
<th>Dose Level</th>
<th>Route of Administration</th>
<th>Dosing Period</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carbenoxolone</td>
<td>25 mg/kg/day</td>
<td>Orally in drinking water</td>
<td>7-14 days</td>
</tr>
<tr>
<td>BX 363A</td>
<td>25 mg/kg/day</td>
<td>Orally in drinking water</td>
<td>7 days</td>
</tr>
<tr>
<td>Penicillin G</td>
<td>0.15 mg/kg/day</td>
<td>Orally in drinking water</td>
<td>7 days</td>
</tr>
<tr>
<td>Ethanol</td>
<td>1.9 mg/kg (1 ml 35% W/v)</td>
<td>Intubation</td>
<td>single dose, sacrificed after 1 hour</td>
</tr>
<tr>
<td>Indomethacin</td>
<td>10 mg/kg (0.2 ml 3% aq. ethanol)</td>
<td>Intubation</td>
<td>&quot; &quot; &quot; &quot; &quot; &quot;</td>
</tr>
<tr>
<td>Prostaglandin $E_1$</td>
<td>1.5 mg/kg (0.2 ml 3% aq. ethanol)</td>
<td>Intubation</td>
<td>&quot; &quot; &quot; &quot; &quot; &quot;</td>
</tr>
<tr>
<td>Prostaglandin $E_2$</td>
<td>1.5 mg/kg (0.2 ml 3% aq. ethanol)</td>
<td>Intubation</td>
<td>&quot; &quot; &quot; &quot; &quot; &quot;</td>
</tr>
<tr>
<td>15-R-15 methyl PGE$_1$ methyl ester</td>
<td>1.5 mg/kg (0.2 ml 3% aq. ethanol)</td>
<td>Intubation</td>
<td>&quot; &quot; &quot; &quot; &quot; &quot;</td>
</tr>
<tr>
<td>16,16-dimethyl PGE$_2$</td>
<td>1.5 mg/kg (0.2 ml 3% aq. ethanol)</td>
<td>Intubation</td>
<td>&quot; &quot; &quot; &quot; &quot; &quot;</td>
</tr>
<tr>
<td>AY22469</td>
<td>1.5 mg/kg (0.2 ml 3% aq. ethanol)</td>
<td>Intubation</td>
<td>&quot; &quot; &quot; &quot; &quot; &quot;</td>
</tr>
</tbody>
</table>

Control animals received vehicle only delivered by the same route as the test solutions.
Table 3.7 Effects of carbenoxolone, BX363A, penicillin G, ethanol and indomethacin on the incorporation of N-acetyl-[\textsuperscript{3}H]glucosamine into an acid-precipitable fraction of rat gastric mucosal scrapings

<table>
<thead>
<tr>
<th>Compound</th>
<th>Incorporation Rate</th>
<th>% change from control</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>nmol x 10\textsuperscript{-4}/mg glycoprotein/4h.</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>Treated</td>
<td></td>
</tr>
<tr>
<td>Carbenoxolone (7 days)</td>
<td>58.82 ± 4.76(6)</td>
<td>85.28 ± 5.20(6)</td>
</tr>
<tr>
<td>Carbenoxolone (14 days)</td>
<td>56.44 ± 6.84(6)</td>
<td>178.76 ± 47.36(6)</td>
</tr>
<tr>
<td>BX363A</td>
<td>48.02 ± 4.10(6)</td>
<td>64.75 ± 1.62(6)</td>
</tr>
<tr>
<td>Penicillin G</td>
<td>64.26 ± 4.74(5)</td>
<td>8.24 ± 1.58(5)</td>
</tr>
<tr>
<td>Ethanol</td>
<td>54.78 ± 3.11(6)</td>
<td>16.39 ± 0.82(6)</td>
</tr>
<tr>
<td>Indomethacin</td>
<td>31.24 ± 5.36(6)</td>
<td>66.18 ± 7.79(6)</td>
</tr>
</tbody>
</table>

Results are shown as means ± S.E.M. of the number of observations shown in parentheses.

Students' t-test * P < 0.05, ** P < 0.01, *** P < 0.001.
of 87% and 70% respectively. Associated with this decrease haemorrhagic areas were observed in all of the stomachs from rats treated with ethanol and in the stomachs of five of the six rats treated with penicillin G. Haemorrhagic areas were also seen in the stomachs of all of the rats treated with indomethacin. In this case, however, the erosions were not associated with a decrease but an increase in the incorporation rate of 112%.

Effects of Ethanol on the Incorporation of N-acetyl-[3H]-glucosamine into acid precipitable glycoproteins during a five hour period after dosing

The effect of ethanol on glycoprotein synthesis in the gastric mucosa was further studied by determining the rate of incorporation of N-acetyl-[3H]-glucosamine, 1, 2, 3, 4 and 5 hours after a single dose (1.9mg/kg, 1ml 35% v/v by intubation). Mucosal preparations from animals which had received a similar amount of water by intubation were assayed at the same time intervals. The results are shown in Table 3.8 and Fig. 3.7.

It can be seen that alcohol pretreatment caused a significant reduction of 61% within one hour of dosing. The percentage change from the controls however decreased up to the third hour from dosing when the inhibition was only 4%. After this time the difference increased until after five hours there was a significant inhibition (67%) in the rate of incorporation. This reduction in the percentage change from the controls is due to a decrease in incorporation by the control preparations, in which the rate of incorporation was reduced significantly after 2 hours compared to the first and fifth hour, and also to an apparent increase in incorporation into mucosal preparations from alcohol treated rats in which the rate at 3 hours was significantly
<table>
<thead>
<tr>
<th>Time from dosing</th>
<th>Incorporation Rate</th>
<th>Change from control %</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Alcohol Treated</td>
</tr>
<tr>
<td>1 hr</td>
<td>50.12 ± 4.07(5)</td>
<td>19.46 ± 4.93(5)**</td>
</tr>
<tr>
<td>2 hr</td>
<td>35.45 ± 3.37(5)</td>
<td>26.78 ± 7.34(5)</td>
</tr>
<tr>
<td>3 hr</td>
<td>37.98 ± 4.59(5)</td>
<td>36.27 ± 5.63(5)</td>
</tr>
<tr>
<td>4 hr</td>
<td>39.07 ± 5.75(5)</td>
<td>29.62 ± 3.59(5)</td>
</tr>
<tr>
<td>5 hr</td>
<td>53.43 ± 6.31</td>
<td>17.81 ± 4.07(5)**</td>
</tr>
</tbody>
</table>

Results are shown as means ± S.E.M. of the number of observations shown in parentheses.

Students 't' test: ** P < 0.01; *** P < 0.001.

Control preparations: Rate at 2h V 1 and 5h P < 0.05.
Alcohol treated: Rate at 3h V 1 and 5h P < 0.05.
Fig. 3.7 Effects of ethanol on the incorporation of N-acetyl-[\(^3\)H]-glucosamine 1, 2, 3, 4 and 5 hours after dosing

Results are shown as means ± SEM of five observations. Open triangles represent control animal incorporation, closed triangles mucosa from treated animals.
greater than those at one and five hours.

Effect of prostaglandin pretreatment on the rate of incorporation of N-acetyl-[3H]-glucosamine into rat gastric mucosal scrapings

Prostaglandins $E_1$, $E_2$, 15-R-15 methyl PGE$_2$ methyl ester, 16,16 dimethyl PGE$_2$ and AY22469 were administered to rats as indicated in Table 3.6. The animals were sacrificed five hours after dosing and the incorporation assay carried out. The results are shown in Table 3.9.

All of the prostaglandins, with the exception of PGE$_1$ caused significant increases in the rate of uptake of N-acetylglucosamine. The most potent prostaglandin in this system was the synthetic analogue of PGE$_2$, 16,16 dimethyl PGE$_2$ which resulted in a 63% increase in incorporation rate over the controls. This compound is followed in order of potency by PGE$_2$, 15-R-15 methyl PGE$_2$ methyl ester and AY22469 with increases of 52%, 50% and 22% respectively. Prostaglandin E$_1$ showed an increase of 30% over controls but this trend did not achieve statistical significance.

The Effects of anti-ulcerogenic and ulcerogenic drugs separately and in combination on the rate of incorporation of N-acetyl-[3H]-glucosamine into an acid-precipitable fraction of rat gastric mucosa

The effects of carbenoxolone, PGE$_1$ and PGE$_2$ separately and in combination with indomethacin on the uptake of hexosamine were determined in rats starved for 24 hours in three separate experiments.

Carbenoxolone was administered for seven days in the drinking water (25mg/kg/day), control rats received water only. For the last 24 hours of this dosing period food was withdrawn from both control
Table 3.9. Effects of prostaglandins on the incorporation of $\text{N-acetyl}[^3\text{H}]\text{glucosamine}$ into an acid-precipitable fraction of rat gastric mucosal scrapings.

<table>
<thead>
<tr>
<th>Prostaglandin</th>
<th>Incorporation Rate (nmol x 10^-4/mg glycoprotein/4h)</th>
<th>Change from control (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Drug Treated</td>
</tr>
<tr>
<td>PGE$_1$</td>
<td>38.98 ± 4.08(6)</td>
<td>50.61 ± 7.0 (6)</td>
</tr>
<tr>
<td>PGE$_2$</td>
<td>38.98 ± 4.08(6)</td>
<td>59.43 ± 4.07(6)</td>
</tr>
<tr>
<td>15-R-15 methyl PGE$_2$ methyl ester</td>
<td>42.83 ± 2.15(6)</td>
<td>64.07 ± 2.83(6)</td>
</tr>
<tr>
<td>16,16 dimethyl PGE$_2$</td>
<td>42.83 ± 2.15(6)</td>
<td>69.83 ± 3.05(6)</td>
</tr>
<tr>
<td>AY 22469</td>
<td>46.81 ± 1.59(6)</td>
<td>57.14 ± 1.06(6)</td>
</tr>
</tbody>
</table>

Results are shown as means ± S.E.M. of the number of observations shown in parentheses.

Students' $t$-test $* P < 0.05$, $** P < 0.01$, N.S. not significant.
and treated animals. Prior to killing, half the rats in each group received indomethacin (10mg/kg, 0.2ml 3% v/v ethanol) by intubation. The remaining animals were intubated with solvent only.

Prostaglandins E₁ and E₂ (0.15mg/kg in 0.2ml 3% v/v ethanol) and indomethacin (10mg/kg in 0.2ml 3% v/v ethanol) were administered to rats which had been previously starved for 24 hours. Further groups of animals were dosed simultaneously with PGE₁ and indomethacin (0.15mg/kg and 10mg/kg respectively in 0.2ml 3% v/v ethanol) and PGE₂ and indomethacin at the same dose levels. Control animals were intubated with 3% v/v ethanol (0.2ml) only. Animals were killed 5 hours after dosing and the incorporation assays carried out. The results of these studies are shown in Tables 3.10 3.11 and 3.12.

The control rates of incorporation in these studies were very much lower than the control rates seen in comparable experiments, 4.44±0.17 compared to 42.87±1.31 n mol x 10⁻⁴/mg glycoprotein/4h, in which a 24 hr starvation period was not used. These results indicate that starvation is a potent inhibitor of the incorporation of hexosamine.

In previous experiments in vivo dosing with PGE₁, PGE₂, carbenoxolone and indomethacin resulted in increased rates of incorporation of N-acetylglucosamine into acid precipitable glycoproteins. In these studies with starved rats the increase caused by carbenoxolone was greatly reduced (increase of 12% over controls) and did not achieve a statistically significant difference.

The increase in uptake of N-acetylglucosamine after indomethacin treatment was again accompanied by the appearance of haemorrhagic areas in the stomach. The appearance of these erosions was prevented by simultaneous administration of PGE₂. Also the rate of incorporation
Table 3.10  The effects of PGE$_1$ and indomethacin separately and in combination on the incorporation of N-acetyl$[^3]$H$]glucosamine into an acid precipitable fraction of rat gastric mucosa

<table>
<thead>
<tr>
<th>Drug treatment</th>
<th>Incorporation Rate nmol x 10$^{-4}$/mg glycoprotein/4h</th>
<th>Change from control</th>
<th>Presence of haemorrhagic areas</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Drug treated</td>
<td></td>
</tr>
<tr>
<td>PGE$_1$</td>
<td>4.53 ± 0.38(3)</td>
<td>5.97 ± 0.18(3) N.S.</td>
<td>+32%</td>
</tr>
<tr>
<td>Indomethacin</td>
<td>4.53 ± 0.38(3)</td>
<td>6.04 ± 0.98(3) N.S.</td>
<td>+33%</td>
</tr>
<tr>
<td>PGE$_1$ + Indomethacin</td>
<td>4.53 ± 0.38(3)</td>
<td>3.56 ± 0.51(3) N.S.</td>
<td>-21%</td>
</tr>
</tbody>
</table>

Results are shown as means ± S.E.M. of the number of observations shown in parentheses.

Students' t-test N.S. not significant.
Table 3.11 The effects of PGE<sub>2</sub> and indomethacin separately and in combination on the incorporation of N-acetyl[<sup>3</sup>H]glucosamine into an acid precipitable fraction of rat gastric mucosa

<table>
<thead>
<tr>
<th>Drug treatment</th>
<th>Incorporation Rate</th>
<th>Change from control</th>
<th>Presence of haemorrhagic areas</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>nmol x 10&lt;sup&gt;-4&lt;/sup&gt;/mg glycoprotein/4h</td>
<td>control</td>
<td>Drug treated</td>
</tr>
<tr>
<td>PGE&lt;sub&gt;2&lt;/sub&gt;</td>
<td>3.97 ± 0.24(3)</td>
<td></td>
<td>5.45 ± 0.18(3)**</td>
</tr>
<tr>
<td>Indomethacin</td>
<td>3.97 ± 0.24(3)</td>
<td></td>
<td>6.22 ± 0.23(3)**</td>
</tr>
<tr>
<td>PGE&lt;sub&gt;2&lt;/sub&gt; +</td>
<td>3.97 ± 0.24(3)</td>
<td></td>
<td>3.95 ± 0.21(3) N.S.</td>
</tr>
<tr>
<td>Indomethacin</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Results are shown as means ± S.E.M. of the number of observations shown in parentheses.

Students’ t-test, ** P < 0.01. N.S. not significant.
Table 3.12  The effects of carbenoxolone and indomethacin separately and in combination on the incorporation of N-acetyl[^H]glucosamine into an acid precipitable fraction of rat gastric mucosa.

<table>
<thead>
<tr>
<th>Drug treatment</th>
<th>Incorporation Rate ( \text{nmol} \times 10^{-4}/\text{mg glycoprotein}/4\text{h.} )</th>
<th>Change from control</th>
<th>Presence of haemorrhagic areas</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Drug treated</td>
<td></td>
</tr>
<tr>
<td>Carbenoxolone</td>
<td>( 5.01 \pm 0.31(3) )</td>
<td>( 5.62 \pm 0.12(3) )N.S. (+12%)</td>
<td>0/3</td>
</tr>
<tr>
<td>Indomethacin</td>
<td>( 5.01 \pm 0.31(3) )</td>
<td>( 6.79 \pm 0.31(3) )**  (+36%)</td>
<td>3/3</td>
</tr>
<tr>
<td>Carbenoxolone + Indomethacin</td>
<td>( 5.01 \pm 0.31(3) )</td>
<td>( 8.07 \pm 0.19(3) )** **  (+61%)</td>
<td>3/3</td>
</tr>
</tbody>
</table>

Results are shown as means ± S.E.M. of the number of observations shown in parentheses.

Students' \('t\)-test: ** \( P < 0.01 \), *** \( P < 0.001 \), N.S. not significant.

Comparison of indomethacin treated with indomethacin and carbenoxolone treated, \( P < 0.01 \).
by these stomachs was at the control level. The results with PGE$_1$ were again inconclusive. Simultaneous administration with indomethacin prevented erosion formation in one of the three stomachs and resulted in a reduction (21%) in the incorporation rate of N-acetylglucosamine. This reduction was not statistically significant.

In contrast to PGE$_2$ and PGE$_1$, a combination of carbenoxolone and indomethacin resulted in an incorporation rate that was significantly greater than the control rate and the rate after indomethacin treatment. In this experiment, seven days pretreatment of rats with carbenoxolone did not prevent the appearance of gastric erosions caused by indomethacin.

Analysis of Radiolabelled Rat Gastric Mucosal Glycoprotein By Concanavalin A-Sepharose and Sephadex G-200 Gel Chromatography

The water soluble component of rat gastric mucosal glycoprotein, purified by affinity chromatography on Concanavalin A-sepharose, was shown to consist of two distinct components when chromatographed on Sephadex G-200 (Fig. 3.8). These components had peak elution volumes of 55ml and 96ml. Using the calibration curve for the column (Fig. 3.9) it can be seen that the first peak had an elution volume similar to that of Blue Dextran 2000 (M.W. 2x10$^6$) which is totally excluded by the Sephadex gel. The molecular weight of this component is therefore in excess of one million. The lower molecular weight component has an apparent molecular weight in the order of 120,000.

The extent of incorporation of N-acetyl-$[^3]$H-glucosamine into the two peaks was found to be identical. The specific activity of the glycoprotein eluted between 39 and 60ml in the first peak was 44±0.91 dpm/μg and that eluted between 81 and 117ml in the second
Fig. 3.8  Analysis of Con A-Sepharose purified glycoprotein from control incorporation by Sephadex G-200

Results plotted as radioactivity per 3ml fraction (open circles) and protein per 3ml fraction (closed circles)
Fig. 3.9 Plot of \( \text{Ve/Vo} \) against log molecular weight for cytochrome c, ovalbumin, bovine serum albumin and alcohol dehydrogenase (yeast) using sephadex G-200.
Fig. 3.10  Analysis of Con-A-Sepharose purified glycoprotein from carbenoxolone treated rats by Sephadex G-200 chromatography

Results plotted as radioactivity per 3ml fraction (open circles) and protein per 3ml fraction (closed circles).
Two peaks with identical elution volumes were also seen (Fig. 3.10) when purified water soluble glycoprotein from the stomachs of rats treated with carbenoxolone (25mg/kg/day for seven days) was applied to the G-200 column. These again corresponded to a high molecular weight molecule (greater than one million) and a lower molecular weight molecule (approx. 120,000). The specific activities for both components were again similar, 103±4.5 dpm/µg (fractions 39 - 54ml) in the first peak and 107±3.98 dpm/µg (fractions 75 - 111ml) in the second. The specific activity of this water soluble glycoprotein from carbenoxolone treated rats is however considerably higher than that seen with control animals.

Hydrolysis of Purified Water Soluble Rat Gastric Glycoprotein from Control Incorporation Study

Thin layer chromatography of the hydrolysis products of water soluble glycoprotein isolated after N-acetyl-[\(^{3}\)H]-glucosamine incorporation, showed (Table 3.13) the presence of galactose, glucosamine, galactosamine and N-acetylneuraminic acid. Radioactivity from N-acetyl-[\(^{3}\)H]-glucosamine was found associated not only with the isolated glucosamine spots but also with galactosamine and N-acetylneuraminic acid. Radioactivity was also seen in another component of the sialic acid hydrolysis ($R_f$ 0.32 in TLC system IV). It is possible that this spot could represent N-glycolneuraminic acid. In contrast no radioactivity from incorporated N-acetyl-[\(^{3}\)H]-glucosamine was found in the neutral sugar hydrolysates.
Table 3.13  Identification of Radiolabelled sugars in hydrolysis mixtures of rat gastric mucosal glycoprotein

<table>
<thead>
<tr>
<th>Sugar</th>
<th>T.L.C. System</th>
<th>Rf.</th>
<th>Presence in hydrolysate</th>
<th>Radioactive</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>I</td>
<td>0.39</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Galactose</td>
<td>I</td>
<td>0.51</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Glucose</td>
<td>II</td>
<td>0.45</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Galactose</td>
<td>II</td>
<td>0.40</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Glucosamine</td>
<td>II</td>
<td>0.23</td>
<td>+</td>
<td>+++</td>
</tr>
<tr>
<td>Galactosamine</td>
<td>II</td>
<td>0.19</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Glucosamine</td>
<td>III</td>
<td>0.48</td>
<td>+</td>
<td>+++</td>
</tr>
<tr>
<td>Galactosamine</td>
<td>III</td>
<td>0.29</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>N-acetyl-neuraminic acid</td>
<td>IV</td>
<td>0.42</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

Details of T.L.C. systems given in Table 3.
3.3. DISCUSSION

The studies reported in this chapter have shown that glycoprotein synthesis in the rat stomach may be studied in vitro over incubation periods of at least four hours by following the incorporation of radiolabelled precursors into an acid precipitable fraction of gastric mucosal scrapings. Other similarities between incorporation into gastric discs (Chapter 2) and mucosal scrapings were also apparent. In both cases uptake of sugar could be inhibited by cycloheximide (10^{-4} M). The optimum concentration of l-glutamine in the incubation medium determined as 8mM for gastric discs by Shillingford (1975) was also found to be optimal for mucosal scrapings.

Despite these similarities, however, several important differences between the two techniques were observed. Although incorporation of hexosamine into acid precipitable glycoproteins of both gastric disc and mucosal preparations was inhibited by cycloheximide the degree of inhibition varied. Whereas a 35% inhibition occurred with discs an inhibition of 51% was apparent in mucosal scrapings after four hours of incubation. This difference implies that the mucosal preparation constitutes a more sensitive method for the determination of the in vitro effects of compounds on glycoprotein synthesis. The increased sensitivity may be due to a greater accessibility of the mucosal cells towards the compound under study in a mucosal cell suspension.

The disc and mucosal preparations also differed with regards to the time dependency of incorporation. The 'lag phase' of one hour observed in disc preparations before the relationship between time and incorporation became linear did not occur when mucosal scrapings were used. Again this difference may result from absorption being facilitated in a free cell preparation.
The most striking difference between the two incorporation systems was in the magnitude of the incorporation rates. In the rat stomach, using gastric discs, a rate of uptake of N-acetyl-[\(^{3}\)H]-glucosamine of \(32\pm 8 \text{ moles} \times 10^{-15}/\text{mg glycoprotein/h}\) was seen compared to \(1460\pm 13 \text{ moles} \times 10^{-15}/\text{mg glycoprotein/h}\) (mean ± S.E.M. of 22 separate experiments) when mucosal scrapings were used. This increase of almost 50 fold must be due at least in part to the nature of the glycoprotein fraction isolated from the two preparations. Unlike that precipitated from gastric discs the fraction precipitated from mucosal scrapings contains macromolecules of mucosal origin only. The absence of muscular and connective tissue contaminants in this precipitate will result in the isolation of a fraction richer in mucosal glycoprotein and hence the observation of a higher rate of incorporation when expressed per mg glycoprotein. The same effect may be responsible for the greatly reduced scatter of results and consequently smaller standard errors of means generally noticed with mucosal scraping incorporations.

The isolation of a purer glycoprotein fraction cannot be wholly responsible for the 50 fold increase in the rate of uptake of hexosamine since the usual quantity of acid precipitable material from disc preparations (approximately 60 mgs.) is only six times the amount derived from scrapings (approximately 10 mgs.). Thus the actual rate of incorporation into mucosal scrapings must be higher. A higher effective concentration of N-acetyl-[\(^{3}\)H]-glucosamine, although equal concentrations were used in both systems, in the vicinity of the glycosylation sites due to the removal of diffusion barriers may be responsible for this increase. Similarly oxygen which was shown to be necessary for incorporation will be more easily available in the homogenised than in the intact preparation.
Thus it can be argued that the incorporation of labelled precursors into mucosal scrapings constitutes a more accurate reflection of glycoprotein synthesis in the stomach than does the use of gastric discs. The increased sensitivity should make it possible to observe subtle drug induced changes in glycoprotein synthesis which would remain undetected in the cruder disc preparation.

That the gut microflora do not contribute to the measured incorporation was proven by the observation that the uptake of labelled hexosamine by gastric washings was insignificant, and cycloheximide, at a concentration inhibitory to growth and metabolism of yeasts (which constitute a large proportion of the rat stomach flora) had no effect on the incorporation rate. In similar incorporation studies using sheep colonic mucosal scrapings (Allen and Kent, 1968) and rat small intestinal slices (Lukie and Forstner, 1972) it has been shown that the microflora in these organs do not play a significant role in the uptake of labelled sugars during the first four hours of incubation.

Addition of penicillin G to the incubation medium did lead to a reduction (30%) in the incorporation rate after 4h incubation. However administration of this drug to animals for 7 days prior to killing also caused a marked reduction in the incorporation rate coupled with the appearance of erosions in the stomachs of the treated animals. It is therefore likely that the decrease in incorporation caused by addition of the compound in vitro is due to an effect on mucosal glycoprotein synthesis rather than an inhibition of bacterial growth.

That N-acetyl-[^3H]-glucosamine is incorporated into glycoproteins was demonstrated by the studies using Concanavalin A-Sepharose and
Sephadex G-200 chromatography in which the isolated radioactivity coincided with protein concentration. The label although mainly associated with N-acetylglucosamine was also shown to be present as N-acetylneuraminic acid and N-acetylgalactosamine. The incorporation of N-acetylglucosamine as sialic acid has previously been demonstrated in studies on the biosynthesis of tracheal mucins in the rat (Gallagher, 1976) and such interconversions are compatible with the scheme for the biosynthesis of aminated precursors of glycoprotein oligosaccharides shown in Fig. 3.11.

It is also theoretically possible for N-acetylglucosamine to be converted to neutral sugars and incorporated as such. However, the results obtained indicate that this does not occur in the present assay. Such an interconversion would require deacetylation and deamination of N-acetylglucosamine-6-phosphate. The deamination reaction:

\[
\begin{align*}
\text{Glutamate} & \quad \text{Fructose-6-phosphate} \\
\text{Glucosamine-6-phosphate} & \quad \text{Glutamine}
\end{align*}
\]

is unlikely to occur in the incubation medium to any great extent due to the high concentration of glutamine (8mM) present.

The elution volumes of the two components of water soluble gastric mucosal glycoprotein on Sephadex G-200 suggest that they have molecular weights in excess of $1 \times 10^6$ and in the region of $1.2 \times 10^5$. Similar results were obtained by Allen and Snary (1972) who isolated glycoprotein fractions of molecular weight $2 \times 10^6$ and $1.1 \times 10^5$ from the water soluble mucosubstance of porcine gastric mucosa by caesium chloride density centrifugation. In the pig these high and low molecular weight glycoproteins had similar chemical analyses, blood group activities and biosynthetic patterns (Snary and Allen, 1971, 1972) and
Figure 3.11  Amino sugar synthesis and activation in glycoprotein biosynthesis
these authors have suggested that the high molecular weight glycoprotein is composed of repeating units of the low molecular weight glycoprotein. The finding that both high and low molecular weight fractions of water soluble mucosubstance from the rat had similar specific activities when labelled with N-acetyl-[\textsuperscript{3}H]-glucosamine indicates that the same situation might occur in this species.

It was shown that other sugars such as glucosamine, glucose, galactose and galactosamine were incorporated into acid precipitable glycoproteins but at markedly lower rates than N-acetylglucosamine. A number of reasons can be forwarded to explain this decreased level of uptake.

(I) Differences in activity of specific glycosyl transferases. N-acetylglucosaminyl transferase may be the most active or abundant glycosyltransferase present in the mucosa.

(II) The enzymes required to form the activated derivative may not be present. The direct formation of UDP galactose from galactose-1-phosphate and UTP is not of major significance in mammalian tissues. The formation of this derivative is through the transfer of the UDP group of UDP-glucose to galactose-1-phosphate. Thus the incorporation of galactose will be dependent on the levels of UDP-glucose in the homogenate.

(III) The position of the sugar in the glycoprotein biosynthesis chain. Glucosamine requires N-acetylation (Fig. 3.11) prior to incorporation and is therefore one step behind N-acetylglucosamine.

and (IV) The participation of the sugars in other metabolic pathways. This factor is of particular relevance to glucose and consequently galactose. Snary and Allen (1972) found that approximately 10% of
the added radioactivity was released as CO₂ during the incorporation of [¹⁴C]-glucose into pig gastric mucosal scrapings.

In the presence of higher concentrations of the sugars the difference between N-acetylglucosamine and glucosamine was absent suggesting that the N-acetylglucosamine pathway is saturated at 5 x 10⁻⁵M concentrations. This saturation may occur in the synthesis of activated precursors, at the glycosyltransferase level or in the availability of acceptors for the sugar precursors. However, studies both with high and low concentrations of sugar showed N-acetylglucosamine to be the best precursor for studying glycoprotein synthesis because of its rapid incorporation and its single metabolic fate i.e. inclusion into oligosaccharides.

It was not possible to distinguish between ulcerogenic and anti-ulcerogenic compounds by their effects on incorporation of N-acetylglucosamine when they were added in vitro to the incubations. Although indomethacin, ethanol and penicillin G, in accordance with their mucosal damaging properties, resulted in a decreased incorporation of N-acetylglucosamine the antiulcerogenic prostaglandins and carbenoxolone failed to cause an increase.

The prostaglandin effects, ranging from slight inhibition (PGE₁ and PGE₂) to no effect (16,16 dimethyl PGE₂ and 15-R-15 methyl PGE₂) to slight stimulation (AY22469), coupled with the absence of a dose related effect suggest that the prostaglandins have no direct effect on the individual enzymes involved in glycoprotein synthesis. Similarly the absence of any in vitro effect over a wide range of concentrations of cyclic AMP (which has been suggested as a secondary messenger in prostaglandin activity) shows that prostaglandin induced increases in mucus synthesis (Fung et al, 1974) are not mediated through an
affect of cyclic AMP on the synthesising enzymes.

The studies with carbenoxolone showed that this compound may require normal subcellular organisation or a greater exposure time than is possible in the in vitro incorporation assay to exhibit a stimulatory effect on incorporation. The concentration dependent inhibition of glycoprotein synthesis observed may be related to the potent uncoupling of oxidative phosphorylation reported for carbenoxolone in vitro (Lindup, 1971).

The only compound which caused a marked increase in incorporation of hexosamines when added to the incubations was L-glutamine, a component of the incubation medium. This increase may be because glutamine is used as an energy source for the biosynthesis of the nucleotide intermediates necessary for glycoprotein synthesis.

Such a role may also be important in considering the ability of glutamine to suppress the development of gastric lesions caused by aspirin (Okabe et al, 1974) and stress (Takagi and Okabe, 1968) which are thought to be due to the development of local ischaemia and disturbances in the energy metabolism of the mucosal cell. However, due to its vital role in the synthesis of amino sugars and sialic acids (Fig. 3.11) it is possible that the protective action of glutamine may be due to a direct stimulatory effect on glycoprotein synthesis. Further evidence in favour of this proposal is given by the finding that aspirin caused an inhibition of glutamine-fructose-6-phosphate transaminase in the rat (Bollet, 1961) and led to a reduction in the concentration of protein bound hexosamines and sialic acid (Menguy and Masters, 1965).

In general a much better correlation between effects on the
incorporation rate of drugs and their ulcerogenic or antiulcerogenic properties was observed when the compounds were administered \textit{in vivo} before the \textit{in vitro} incorporation assay. Carbenoxolone produced an increase in the incorporation rate supporting the hypothesis that its anti-ulcer properties are largely due to an effect on mucus synthesis.

The studies using Sephadex G-200 gel chromatography add further weight to the argument that the effect of carbenoxolone is on the degree of glycosylation of the glycoprotein rather than on the overall amount of glycoprotein synthesised. Pretreatment with the drug resulted in the isolation of two glycoprotein components with higher specific activities than those derived from control mucosal scrapings but did not appear to have any marked effect on the amount of glycoprotein in these peaks.

Similarly it was shown that the stimulatory effects of carbenoxolone appeared to be limited to the incorporation of amino sugars and did not include galactose on which carbenoxolone either had an inhibitory effect at low concentrations or no effect at higher concentrations. As discussed previously (Chapter 2) this differential effect on incorporation rate implies that carbenoxolone results in the production of a modified glycoprotein containing increased amounts of N-acetylglucosamine, N-acetylgalactosamine and N-acetyl-neuraminic acid and less galactose. Further indirect evidence for the production of a glycoprotein with a modified oligosaccharide chain is provided by its gel chromatographic pattern. Although carbenoxolone caused increases in the rate of incorporation of hexosamines in the order of 100% there was no indication of an increase in the molecular weight of the resulting glycoproteins compared to controls.
Similarly the increases in the incorporation rate of N-acetyl-glucosamine seen after pretreatment with prostaglandins which have reported anti-ulcer properties in animal and human studies suggest that these compounds may also exert some of their beneficial action through an effect on mucus synthesis. This conclusion is supported by the work of Fung et al (1974b) who found that 15-R-15 methyl PGE$_2$ methyl ester caused an apparent increase in mucus synthesis in the stomachs of human gastric ulcer patients with a simultaneous increase in the rate of healing of the lesion.

In contrast alcohol and penicillin G led to a decrease in the rate of glycoprotein synthesis associated with the appearance of gastric lesions. Although direct evidence that the primary effect of these compounds is on mucus synthesis is not available, Eastwood and Kirchner (1974) have shown that within 15 min. of dosing with ethanol (10% v/v) the only abnormalities observed in the gastric mucosa of mice by electron and light microscopy were a clumping of nuclear chromatin and a marked decrease in density of the endoplasmic reticulum. The apical membrane, surface mucous granules and mitochondria appeared normal. Primary damage to the endoplasmic reticulum implies that derangement of glycoprotein synthesis, as evidenced by the incorporation studies is an early phenomenon in mucosal damage by ethanol and may subsequently lead to an impaired mucosal barrier, development of erosions and the increase in back diffusion of hydrogen ions, as observed by Davenport (1967).

Factors such as stress and starvation were also shown to have an adverse effect on mucus synthesis. It is an accepted fact that prolonged stress e.g. restraint leads to impaired mucus production
and the appearance of ulceration in animals (Takagi and Yano, 1972). However, studies of incorporation rates during the period immediately following gastric intubation showed that even comparatively minor and acute stressful situations resulted in decreased glycoprotein synthesis for up to five hours. This finding indicates the need for care in the handling of animals used in such studies.

Similarly, in experiments in which a 24h period of starvation was employed, a dramatic decrease in incorporation rates was observed although no mucosal lesions were noted. Similar findings have been reported by MacDonald (1976) and this decreased mucus synthesis may be the cause of the gastric lesions observed after more prolonged periods of starvation (Robert, 1963).

Seemingly anomalous results were produced by indomethacin treatment five hours prior to sacrifice. In treated animals erosions similar to those caused by ethanol and penicillin were observed. In indomethacin treated animals, however, these were accompanied by an increase in the rate of incorporation over the controls. This finding is not consistent with the reports of other authors who have shown indomethacin to cause a decrease in mucus synthesis, both by chemical analysis and in incorporation studies (Menguy and Desbaillets, 1967, Shillingford, 1975). However, these workers examined the effects of indomethacin over considerably longer periods (daily doses for 20 and 7 days respectively) than those used in the present studies and it is possible that the increase seen after acute dosage may represent a stimulation of glycoprotein synthesis in the normal tissue surrounding the lesions due to an 'irritant' effect of the drug.

There are indications that the same phenomena, although to a
lesser extent, may occur with ethanol (Fig. 3.7). The rate of incorporation showed a tendency to increase towards that of the controls within three hours of a single dose.

The inhibitory effects of indomethacin on prostaglandin synthesis have been reported in a large number of animal tissues (Vane, 1971, Dembinska et al, 1974). This antagonism also occurs in the gastric mucosa in which indomethacin has been shown to inhibit prostaglandin synthetase (Main and Whittle, 1975) and consequently physiological events mediated by the prostaglandins, such as mucosal vasodilation (Whittle, 1976). Thus the gastric lesions caused by indomethacin may be the result of antagonism of prostaglandin synthesis and action. This argument is supported by the finding that both the mucosal lesions and the apparent increase in incorporation rate caused by indomethacin were nullified by simultaneous administration of PGE$_2$ and to a lesser extent PGE$_1$.

The presence of endogenous prostaglandins in the stomach and their ability to affect acid secretion and mucosal blood flow have led a number of authors (Vane, 1971, Frankhuijzen and Banta, 1975) to conclude that these compounds may have a physiological role in the maintenance of mucosal homeostasis. The observations that these compounds have a stimulatory effect on the incorporation of N-acetyl-

The inability of carbenoxolone to antagonise the ulcerogenic effect of indomethacin or inhibit the increase in incorporation rate observed after short term treatment with this drug does not preclude the existence of a common mechanism for the anti-ulcer and mucus
stimulating effects of carbenoxolone and the prostaglandins. It has been shown \textit{in vitro} that carbenoxolone while having no effect on prostaglandin synthetase had potent inhibitory effects on prostaglandin 15-hydroxy-dehydrogenase and $\Delta_13$-reductase in guinea-pig lung (Peskar \textit{et al}, 1975) and human gastric mucosa (Peskar, 1975). Inhibition of the enzymes responsible for the breakdown of prostaglandins would not be expected to counter the effects of a synthetase inhibitor. Thus it may be argued that at least some of the beneficial effects of carbenoxolone in the treatment of gastric ulceration may be through potentiation of endogenous E-type prostaglandins.
CHAPTER FOUR
THE EFFECT OF CARBENOXOLONE ON LYSOSONAL STABILITY
4.1 INTRODUCTION

Lysosomal History, Structure and Function

The name 'lysosomes' has been given to a special group of cytoplasmic particles, first identified and named by De Duve and his fellow workers (1955). De Duve's original studies were with rat liver, but lysosomes are now known to occur in most animal tissues, including the stomach (Ayulo and Forest, 1974).

Although lysosomes differ from tissue to tissue (Dingle, 1963) and between cells of the same tissue (Novikoff, 1963) certain generalizations apply. They are about 0.5 microns in diameter and are bound by a single outer membrane, possessing the dimensions and appearance expected for a cellular lipoprotein membrane.

These cytoplasmic vesicles contain some forty hydrolytic enzymes, which exert maximal activity at acid pH and are capable of digesting all the categories of macromolecules in the cell; polysaccharides, proteins, glycoproteins, nucleic acids and lipids. The major enzymes associated with lysosomes are shown in Table 4.1.

Formation of lysosomes containing digestive enzymes and substrate is preceded by two separate events. In the first, small hydrolase-rich sacs pinch off from the Golgi apparatus and give rise to primary lysosomes. In the second, infoldings of the cell membrane form vacuoles containing intracellular material which is now endocytosed. These vacuoles (endocytic vacuoles) are the avenue to the interior of the cell for substances which cannot cross the cell membrane (e.g. proteins). A primary lysosome and an endocytic vacuole can then fuse and acquire a common membrane. The new hybrid vacuole (secondary lysosome) now
| Lipids         | Acid lipase  |
|               | Ceramidase   |
|               | Phospholipases A₁ and A₂ |
|               | Sphingomyelinase |
|               | Esterases (active against thiol, indoxyl-, naphthyl, fatty acid and cholesterol esters) |
| Nucleic Acids | Acid deoxyribonuclease |
|               | Acid ribonuclease |
|               | Acid phosphatase |
|               | Acid pyrophosphatase |
|               | Phosphodiesterase |
| Complex lipids| N-acetyl-α-galactosaminidase |
| Polysaccharides| N-acetyl-α-glucosaminidase |
| Glycoproteins | N-acetyl-β-hexosaminidase |
|               | Aspartylglucosylaminidase |
|               | Fucosidase(s) |
|               | Galactosidase (α and β) |
|               | Glucosidases (α and β) |
|               | Hyaluronidase |
|               | Iduronidase |
|               | Mannosidases (α and β) |
|               | Neuraminidase |
|               | O-Seryl-N-acetyl-α-galactosaminidase |
|               | β-xylosidase |
| Proteins      | Acid carboxypeptidase |
|               | Amino acid napthylaminidase |
|               | Cathepsin A (probably carboxypeptidase) |
|               | Cathepsin B (B₁ and B₂; endopeptidases) |
|               | Cathepsin C (dipeptidylaminopeptidase) |
|               | Cathepsin D (acid "proteinase") |
|               | Dipeptidylaminopeptidase II |
contains endocytosed material and lysosomal hydrolases in close contact and subsequently the former becomes the substrate for the latter (De Duve and Wattiaux, 1966). Intracellular materials to be digested are disposed of by the process of autophagy (De Duve and Wattiaux, 1966) in which part of the cell's cytoplasm becomes segregated by a membrane which surrounds it and separates it from the rest of the cell. Here too the autophagic vacuole formed fuses with a primary lysosome and catabolism can take place.

Catabolism within the lysosome usually means breakdown of the material to the simplest units, i.e. amino acids and sugars from glycoproteins, mononucleotides from nucleic acids and simple lipids and sugars from glycolipids. Catabolic products of a molecular weight smaller than 200 can usually diffuse out through the lysosomal membrane (Gorden, 1973) and be re-utilised by the cell. Non-digestible remnants that cannot diffuse can be expelled by exocytosis which involves fusion of the secondary lysosome with the cell membrane and exteriorization of the lysosomal contents.

Lysosomes appear to be involved in a large number of normal physiological processes. Weber (1963) has shown that in the tail of the developing tadpole of the South African frog (Xenopus laevis) the levels of cathepsin are elevated and increase as the tail regresses until the stump contains large amounts of lysosomal enzymes and drops off. A similar mechanism is thought to apply to the resorption of bone by osteoclasts (Vaes, 1969). According to this view the hydrolases are excreted in bulk, by exocytosis, in the intracellular resorption zone near the osteoclasts, where they exert an eroding action on the organic components of the bone matrix. Fragments released from the matrix by
this process may be taken up in osteoclasts by pinocytosis to be further digested intracellularly.

Lysosomes play a major role in the catabolism of intracellular (Segal, 1975; Dean, 1975) and extracellular (Gregoriadis, 1975) proteins and glycoproteins. The gastrointestinal tract has been proposed as a major site for the degradation of glycoproteins (Hsu and Tappel, 1965; Robinson, 1969) and it has been suggested that part of their role in these organs is the digestion of sloughed mucus and epithelial cells to provide substrates for the synthesis of new cells and mucus (Gordis, 1966).

Degradation of glycoproteins, the major component of mucus, to individual sugars and amino acids involves the breakage of three types of bond; bonds between any two sugars, bonds between a sugar and amino acid in the polypeptide backbone, and bonds between amino acids. Such a breakdown would require the action of a large number of hydrolases as is found within the lysosomes. Figure 4.1 shows the structure of an imaginary gastric glycoprotein containing galactose, N-acetyl-galactosamine, N-acetylglucosamine, N-acetylneuraminic acid and fucose, and some of the lysosomal enzymes which would be involved in its breakdown.

In view of the nature of the lysosomal contents it is hardly surprising that lysosomes have been implicated in a number of pathological processes. Involvement in the disease process may either be through a deficiency of one or more lysosomal enzymes, or through the effects on living tissue of hydrolases released via damaged membranes. However, evidence linking lysosomal disruption to disease processes is indirect, being based on experimental evidence, often without clinical confirmation.
Figure 4.1  Hydrolysis of Gastric Glycoproteins by Lysosomal Enzymes

1. Neuraminidase (Yaha and Carubelli, 1967)
2. N-acetyl-α-D-galactosaminidase (Weissmann and Friedorici, 1966)
3. β-D-galactosidase (Sellinger et al., 1960)
4. N-acetyl-B-0-glucosaminidase (Sellinger et al., 1960)
5. α-1-fucosidase (Conchie and Hay, 1963)
6. α-D-galactosidase (Van Hoof and Hers, 1968)
7. Cathepsins (Vaes, 1973)
The best known examples of diseases associated with a deficiency or lack of lysosomal hydrolases (storage diseases) involve lipids, polysaccharides and mucopolysaccharides. Thus, in Tay-Sachs' disease, hexosaminidase A activity is deficient (O'Brien, 1973) and the ganglioside GM₂ accumulates in neurons and elsewhere (Terry, 1971). In metachromic leukodystrophy arylsulphatase A activity is lacking and bodies rich in cerebroside sulphate are prominent in glial cells (Austin, 1973; Eto et al., 1974). In the Hurlers syndrome (gargoylism) \( \alpha \)-1-iduronidase (Bach et al., 1972), \( \beta \)-galactosidase and \( \alpha \)-fucosidase (Van Hoof and Hers, 1968) may be deficient leading to an accumulation of mucopolysaccharides, and in Pompes disease (glycogenosis type II) \( \alpha \)-glucosidase is absent and abnormal deposits of glycogen are found in hepatocytes and muscle cells (Hers and De Barys, 1973). A comprehensive review of inborn lysosomal diseases is available (Hers, 1973).

In various forms of arthritis, cartilage or bone matrices are extensively eroded. Synovial fluids from arthritic joints show elevated levels of extracellular hydrolases (Caygill and Pitkeathly, 1966; Collins and Lewis, 1971). These observations coupled with the findings that acid hydrolases accumulate extracellularly during autolysis of explanted cartilage or bone (Fell and Dingle, 1963) have led to the concept that degradative aspects of the damage to connective tissue are due in large measure to abnormal release of lysosomal enzymes from cells of the perichondrium or synovium, from chondrocytes and from phagocytes that enter the damaged tissue. The involvement of lysosomal enzymes in the arthritic breakdown of connective tissue has been reviewed recently (Dingle, 1973).
The effects of a number of noxious agents on cells have been tentatively attributed to intracellular release of hydrolases as a primary event. Proposals of this type have been made for the cytotoxic actions of bacterial endotoxins (Weissman, 1967) and the killing of cells by photoreactive dyes or silica (Ericsson et al., 1974). Killing of alveolar macrophages by inhaled fibres and subsequent repeated release from dead cells and re-uptake cells could possibly be a major factor in silicosis.

Involvement of Lysosomes in Gastric Ulceration

Another disease state in which lysosomes have been implicated is gastric ulceration. Ferguson et al. (1972) found that gastric ulcers in restrained rats were associated with reduced mucosal levels of cathepsin D, indicating rupture of lysosomal membranes and release of enzymes. The action of these enzymes on protective mucus lining the stomach could be responsible for the 'underglycosylated' glycoprotein apparent in gastric lesions.

Similar evidence exists for lysosomal involvement in drug induced gastric ulceration. Serotonin was found to induce gastric ulcers in rats, associated with a release of lysosomal enzymes (Ferguson et al., 1973) and the non-steroidal anti-inflammatory drug phenylbutazone, at ulcerogenic doses was found to accelerate the breakdown of lysosomes in isolated stomach preparations from the rat and rabbit (Lewis et al., 1971).

Steroidal anti-inflammatory agents such as cortisone and prednisolone have also been shown to damage the gastric mucosa (Takagi and Abe, 1974). It would seem anomalous that the anti-inflammatory steroids and certain
non-steroidal anti-inflammatory agents such as phenylbutazone cause
gastric lesions associated with lysosomal damage, since it is believed
that the beneficial action of these drugs in inflammatory diseases
such as arthritis is due, at least in part, to lysosomal membrane
stabilization (Nakanishi and Gato, 1975). However, the effect on
lysosomal stability has been shown to be concentration dependent
(Lewis et al., 1970; Symons, 1971), causing stabilization at lower
concentrations and lysis at higher concentrations. Lewis (1970) has
suggested that a local build up of drug concentration, following long
term oral treatment and subsequent labilization of membranes may be a
causative factor in the associated ulceration.

The Effect of Carbenoxolone on Lysosomal Stability

In studies using artificial vesicles, liposomes, it has been
shown that carbenoxolone has a biphasic effect on membranes. High
concentrations of the drug (greater than $10^{-3}$ M) cause lysis and lower
concentrations ($10^{-6}$ M) increase the stability of liposomes (Symons,
1976). Studies on the effects of carbenoxolone on the stability of
endogenous vesicle membranes are however lacking.
4.2 MATERIALS AND METHODS

Chemicals

Phenolphthalein glucuronide, p-nitrophenyl phosphate, p-nitrophenyl-N-acetyl-β-D-glucopyranoside and Triton X-100 were obtained from the Sigma Chemical Company Ltd., London. Carbenoxolone sodium was a gift from Biorex Laboratories Ltd., London. All other chemicals were Analar grade wherever possible and obtained from either British Drug Houses Ltd., Poole, Dorset, or the Sigma Chemical Company Ltd., London.

Isolation of a Lysosome Fraction

The method used was a modification of Weissmann's (1965). Four male Wistar albino rats (body weight approx. 300 g) from the University of Surrey Breeding Unit, were killed by cervical dislocation and their livers immediately removed, weighed and placed in ice-cold 0.25M sucrose. Between 30 and 40g of liver were obtained. The liver was then minced finely with scissors (2-3 mm cubes) and 3-5g portions homogenised in 0.25M sucrose using a Potter Elvejém homogeniser. All operations were carried out at 4°C. It was found that two up and down strokes with the pestle were sufficient to form a homogenous suspension. This initial suspension was diluted to a final concentration of 10% w/v with 0.25M sucrose (300-400 ml, total volume).

This whole liver homogenate was centrifuged at 750 g\text{av.} for 10 min. in an MSE Highspeed 18 centrifuge at 4°C. This sedimented the unbroken cells, debris and nuclei leaving the cytoplasmic contents in suspension. The pellet was discarded and the supernatant centrifuged at 20,000 g\text{av.} for 20 min. also in the MSE Highspeed centrifuge, which sedimented a 'large granule' fraction containing unbroken lysosomes. The pellet was washed twice in 150 ml volumes of 0.25M sucrose. This involved careful
resuspension of the pellet and recentrifuging at 20,000 \( g_{av} \) for 20 min. The washed pellet was finally suspended in ice-cold 0.25M sucrose containing 0.05 M tris-HCl buffer (pH 7.4). 2 ml of sucrose-buffer were added for every gram of liver used. This final suspension was used in subsequent experimentation and will be referred to as the 'lysosome' or 'lysosome rich' fraction.

The Effect of Carbenoxolone on Lysosomal Enzyme Release

Portions (5 ml) of the lysosome suspension were added to 50 ml conical flasks, stoppered and shaken in a shaking water bath (Mickel) at 100 oscillations per min. Carbenoxolone was added as a small volume of aqueous solution. Appropriate controls were always run. The time and temperature of incubation was 90 min. at 37°C and the pH of all experiments was 7.4 (tris-HCl buffer).

The level of free enzymes present in fresh lysosome suspensions before incubation was always measured by centrifuging a small volume (5 ml) at 20,000 \( g_{av} \) for 20 min using an MSE Superspeed 50 centrifuge and a 10 x 10 ml rotor and assaying the enzyme activity in the supernatant. In a similar way the amount of free enzyme after any treatment the lysosomal suspension received could also be measured. The total amount of enzyme that could be released from the lysosomes was measured by subjecting them to the lytic action of 0.1% Triton X-100, a non-ionic detergent, and measuring enzyme levels after centrifugation.

Enzyme Assays

(1) Acid phosphatase (orthophosphoric monoester phosphohydrolase)

A modification of the method of Huggins and Talahay (1945) was used. Portions (0.5 ml) of p-nitrophenylphosphate (0.015M) and 0.5 ml Sorensen's citrate buffer, 0.09M, pH 4.8 were mixed together and 100\( \mu \)l of the
supernatant to be assayed added. The reaction mixture was incubated at 37°C for 30 min (100 oscillations/min) after which 5 ml of 0.1 M NaOH were added. This stopped the enzymic reaction and the released p-nitrophenol was determined at 400 nm in a Cecil CE272 spectrophotometer. Appropriate reagent and supernatant blanks together with a p-nitrophenol standard were always run. The standard calibration curve for p-nitrophenol is shown in Fig. 4.2 (a).

(ii) β-Glucuronidase (β-D-glucuronide glucuronohydrolase)

A modification of the method of Talahay et al. (1946) was used. Portions (0.5 ml) of phenolphthalein glucuronide (0.0015M) and 0.5 ml acetate buffer (0.2M, pH 4.5) were mixed together and 100μl of the supernatant to be assayed added. The reaction mixture was incubated at 37°C for 30 min (100 oscillations/min) after which 5 ml of 0.2M glycine buffer, pH 10.4, were added. This stopped the enzymic reaction and developed the colour of released phenolphthalein which was determined at 540 nm. The standard calibration curve for phenolphthalein in shown in Fig. 4.2 (b).

(iii) N-acetyl glucosaminidase (β-2-acetimido-2-deoxy-D-glucoside acetamidodeoxy-glucohydrolase)

This was estimated using p-nitrophenyl-N-acetyl-β-D-glucopyranoside as substrate (Caygill and Jevons, 1965). Portions (0.5 ml) of a 0.0015M solution of the substrate and 0.5 ml of 0.2M acetate buffer, pH 4.5, were mixed. 100μl of the supernatant to be assayed was added. The conditions of incubation and estimation were exactly as for acid phosphatase.

Protein Assay

Suitable dilution of the lysosomal suspensions were routinely measured for their protein content, as an index to which other parameters could be referred. The spectrophotometric method of Lowry et al. (1951)
(a) Extinction (410 nm)

Concn of p-nitrophenol (μg/ml)

(b) Extinction (540 nm)

Concn of phenolphthalein (μg/ml)
Fig. 4.3 Standard calibration curve for the estimation of protein using the method of Lowry et al (1951)

Bovine serum albumin was the protein standard used.
was used with bovine serum albumin as a standard. The standard calibration curve is shown in Fig. 4.3.

4.3 EXPERIMENTAL AND RESULTS

(a) Time course of enzyme release

A lysosomal preparation (60 ml) in 0.05M tris-HCl buffered 0.25M sucrose at pH 7.4 was incubated at 37°C (100 oscillations/min). Samples (5 ml) were removed at various time points, centrifuged and the activity of acid phosphatase in the supernatant determined. Figure 4.4 shows the release of acid phosphatase over a 5-hour period, the activity being expressed as mg p-nitrophenol formed from p-nitrophenyl phosphate per mg protein in the lysosomal fraction. It can be seen that the amount of enzyme released increases with time, the rate of release being greater in the first hour than in the subsequent 4 hours. Even after 5 hours of incubation the level of released enzyme did not reach that which was released by freezing and thawing a sample six times.

Although the results in Figure 4.4 were related to the protein concentration of the lysosomal suspension it was found after further experimentation that the amount of lysosomal enzyme in different lysosomal preparations was in no way proportional to the protein concentration of the fraction. It was therefore necessary to run strict controls with every experiment and for comparison to express all increases or decreases in enzyme activity as a percentage of these controls.

(b) Effect of Triton X-100 on enzyme release

Aliquots (5 ml) of a lysosomal suspension (in 0.05M tris/HCl buffered 0.25M sucrose, pH 7.4) were placed in 50 ml conical flasks. To these was added 50μl of aqueous triton X-100 solutions over a range
ig. 4.4 Time course of acid phosphatase release from a lysosomal fraction

Level reached by freezing and thawing a sample 5 times
Fig. 4.5 The effect of Triton X-100 concentration on the acid phosphatase release from a lysosome fraction
of concentrations to give final concentrations in the flasks of $10^{-6} - 5 \times 10^{-1}$% v/v triton X-100. Appropriate aqueous controls were also run. The flasks were incubated as normal for a period of 90 minutes. Free acid phosphatase levels were measured initially and after incubation. Figure 4.5 shows the results obtained.

Triton X-100 is capable of releasing acid phosphatase from the lysosomes and this effect was concentration dependent. The critical concentration of Triton X-100 appears to be in the region of $10^{-3}$% v/v. Between this concentration and 0.1% v/v triton the lysosomes are very sensitive to concentration changes. Above concentrations of 0.1% very little increase was noted.

The Effects of Carbenoxolone on lysosomal enzyme release

A series of experiments was conducted using a range of carbenoxolone concentrations. The carbenoxolone was added in a small volume of aqueous solutions (50μl) to conical flasks containing 5 ml of lysosomal suspension (in 0.05M tris-HCl buffered 0.25M sucrose, pH 7.4), to give a final concentration range of $10^{-3} - 10^{-8}$M. Aqueous controls were run simultaneously. The flasks were incubated normally, the free levels of acid phosphatase, β-glucuronidase and N-acetyl glucosaminidase being measured before and after incubation. The results are shown in Figures 4.6, 4.7 and 4.8.

It can be seen that carbenoxolone appeared to have a biphasic effect on the stability of rat liver lysosomes as measured by the release of the three enzymes studied. Lysis of the lysosomes leading to increased enzyme leakage occurred with high concentrations ($10^{-4}$M and above). Lower concentrations showed either a stabilizing effect or no significant effect at all. Maximum stabilization always occurred
Figure 4.6 Effect of carbenoxolone on lysosomal stability

(I) Acid phosphatase

Results are plotted as a percentage of control leakage of enzyme (Control=100) and are shown as the mean and SEM of four experiments. Total enzyme releasable by Triton x-100=1152±438%.
Fig. 4.7 Effect of carbenoxolone on lysosomal stability

(II) β-Glucuronidase:

Results are plotted as percentage of control leakage of enzyme (Control=100) and are shown as mean and SEM of four experiments. Total enzyme releasable by Triton x-100 = 932±319%
Fig. 4.6 Effect of Carbenoxolone on lysosomal stability

(III) N-Acetyl glucosaminidase:

Results are plotted as percentage of control leakage of enzyme (Control=100) and are shown as mean and SEM of four experiments. Total enzyme releasable by Triton X-100 = 984±208%
in the region of $10^{-5}$ M carbenoxolone.

The effect of the same range of carbenoxolone concentrations on the thermal stability of acid phosphatase, $\beta$-glucuronidase and N-acetylglucosaminidase was also studied to determine whether this reduction in enzyme levels was due to inhibition of the enzymes rather than a stabilization of the lysosomal membrane preventing or retarding release of the enzymes.

An enzyme rich supernatant was obtained by centrifuging a lysosomal preparation which had been frozen and thawed six times. Portions (2 ml) were incubated at $37^\circ$C with a range of carbenoxolone concentrations ($10^{-8}$-$10^{-3}$ M) water alone being added to control incubations. Enzyme activity of acid phosphatase, $\beta$-glucuronidase and N-acetylglucosaminidase was measured before and after incubation. The results are shown in Table 4.2 expressed as a percentage of the activity in control incubations.

The results show that carbenoxolone tended to cause a reduction in the activity of the three enzymes over the concentration range used. A concentration dependency for this decrease was not apparent but the highest degree of inhibition was always apparent at the highest concentration used, namely $10^{-3}$ M carbenoxolone.
Table 4.2

The effect of carbenoxolone on the thermal stability of acid phosphatase, β-glucuronidase and N-acetyl-glucosaminidase from rat liver lysosomes

<table>
<thead>
<tr>
<th>Carbenoxolone concn. (M)</th>
<th>Enzyme activity after 90 min. incubation (% of control values)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Acid phosphatase</td>
</tr>
<tr>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>10⁻⁸</td>
<td>91</td>
</tr>
<tr>
<td>10⁻⁶</td>
<td>95</td>
</tr>
<tr>
<td>10⁻⁵</td>
<td>91</td>
</tr>
<tr>
<td>10⁻⁴</td>
<td>93</td>
</tr>
<tr>
<td>10⁻³</td>
<td>71</td>
</tr>
</tbody>
</table>

Results are expressed as the means of duplicate samples and have been corrected for the free enzyme activity apparent before incubation.
Fig. 4.9 The effect of carbenoxolone sodium on the stability of rat liver lysosomes

Results corrected for compounds effect on free enzyme activity (Acid phosphatase - closed triangles; β-glucuronidase - closed circles; N-Acetylglucosaminidase - closed squares).
4.4 DISCUSSION

The lysosome preparation used in these experiments was from liver, for reasons of yield per gram of tissue, rather than the stomach, and was impure. No attempt was made to separate mitochondria from lysosomes. The apparatus and techniques needed to successfully accomplish this separation, resulting in a purer and more reproducible preparation are given by Beaufay (1969) and Dingle and Barret (1969). Yet, despite the extremely elaborate procedures used, even these authors have failed to produce a 100% pure lysosomal preparation.

However, despite the impurity of the fraction, specific lysosomal enzymes were studied and it could be argued that the in vitro experiments conducted were at least as relevant to the in vivo situation as a purer lysosomal preparation would have been. The prepared fraction contained a large number of intact lysosomes which could be labilized by incubation at 37°C or to a greater extent by treatment with the non-ionic surfactant Triton X-100.

These studies show that concentration is of critical importance when considering the effects of carbenoxolone on lysosomes. The drug possesses a biphasic effect on lysosomal stability, measured by the release of acid phosphatase, β-glucuronidase and N-acetylglucosaminidase. High concentrations (10^{-4} M and above) caused lysis while lower concentrations showed either a stabilizing effect or no effect at all. At 10^{-5} M concentrations maximum stabilization occurred and although the degree of stabilization was never more than 12-22% at this concentration the results are highly significant.
The inhibitory effect of carbenoxolone on the free lysosomal enzymes must account for some of the observed decrease in enzyme activity otherwise attributable to stabilization. Such an inhibition could be due to carbenoxolone binding to the enzyme protein, perhaps obscuring the active site. When, however, the loss of enzyme activity due to stabilization was corrected for that due to enzyme inhibition a similar pattern is produced for all the enzymes (Figure 4.9). The biphasic effect is still apparent and stabilization, although to a lesser extent, may still be seen at $10^{-5}$M carbenoxolone, higher concentrations of the drug producing an even more marked lytic effect.

There have been a number of reports linking lysosomal enzymes with ulceration of the gastro-intestinal tract. Ulceration of the caecum in guinea pigs by carrageenan has been attributed to lysis of macrophage lysosomes due to ingestion of this compound (Abraham et al., 1974). Similarly, in the stomach gastric damage is associated with a leakage of lysosomal enzymes.

Gastric ulcers in restrained rats are associated with reduced mucosal levels of the enzyme cathepsin D (Ferguson et al., 1972), as are ulcers induced by serotonin (Ferguson et al., 1973), and acute alcohol intoxication which was shown to cause gastric lesions in rats (Chapters 2 and 3) is associated with abnormal fragility of lysosomes and discharge of enzymes (Geokas and Rinderknecht, 1973). Similarly, phenylbutazone and azopropazone were shown to induce gastric lesions associated with lysis of gut lysosomes, the more potent ulcerogenic effect of phenylbutazone being associated with a more marked effect on lysosomal leakage (Lewis et al., 1971). In contrast to
these results prostaglandin $E_1$ which has been shown to have an anti-ulcerogenic effect (Robert et al., 1968) has been shown to decrease the leakage of lysosomal enzymes in the gastric mucosa (Ferguson et al., 1973).

These results do not indicate whether release of lysosomal enzymes is causative in the production of the lesion or merely an indication of tissue damage. Although even if secondary, the release of lysosomal enzymes with their considerable digestive capabilities would aid the development of the lesion. However, in vitro studies have shown that phenylbutazone and azopropazone have a direct lytic effect on isolated liver lysosomes (Lewis et al., 1971). Furthermore, prostaglandin $E_1$ has been shown to have a direct stabilizing effect on lysosomes from human polymorphonuclear leukocytes (Weissman et al., 1971), canine hepatocytes (Raflo et al., 1973) and rat gastric mucosal cells (Ferguson et al., 1973).

Similarly to prostaglandin $E_1$ carbenoxolone was shown to cause a reduction in the release of lysosomal enzymes from rat liver and it may be postulated that such an effect on gastric mucosal lysosomes could play a role in the anti-ulcerogenic action of this drug. Studies showed a stabilization of no greater than 22%. This, however, was in an in vitro situation and the effect under in vivo conditions of pH etc. could be more dramatic. The membrane stabilizing role of carbenoxolone is supported by the work of Symons (1976) who found that $10^{-6}$ M concentrations of the drug resulted in decreased permeability of phosphatidylcholine and phosphatidylcholine:cholesterol liposomes.

A similar stabilizing mechanism has been proposed for the anti-inflammatory corticosteroids used in the treatment of arthritis. These also show a biphasic effect on lysosomal stability (Lewis, 1970; Symons,
Anti-inflammatory drugs have however been implicated in gastric mucosal damage and Lewis (1970) has suggested that this could be due to an accumulation of the drugs in the vicinity of the lysosomes during long term therapy, thereby producing lytic concentrations, lyosomal rupture and ulceration. Consequently it might be expected that carbenoxolone with a lower in vitro lytic concentration, $5 \times 10^{-5} \text{M}$ compared to $5 \times 10^{-4} \text{M}$ (Symons, 1971), would produce gastric lesions in clinical use. However, due to its rapid absorption and high degree of binding to plasma proteins, an average level of $15 \mu \text{g/ml}$ of plasma, equivalent to 75% of a therapeutic dose (100 mg), within two hours of dosing in humans (Baron et al, 1975) it is unlikely that the drug would reach lytic concentrations in the vicinity of the gastric mucosal lysosomes.

Thus it may be postulated that lysosomal fragility is important in the genesis of drug induced ulceration in animals and possibly in chronic gastric ulceration in humans. Whether rupture of these organelles is a primary feature or secondary in aiding the development of the lesion, lysosomal stabilization could in part explain the beneficial action of carbenoxolone in the treatment of this disease. Such a stabilization could explain the decreased cell turnover noted by Lipkin (1970) and may explain in part, the increase in glycoprotein synthesis which occurs after carbenoxolone treatment.
CHAPTER FIVE

GENERAL DISCUSSION
This chapter presents a view of the experimental work carried out, the conclusions drawn from it and suggestions for further work.

In view of the thermal and chemical conditions in the stomach which are optimal for tissue digestion it is pertinent to ask why gastric ulceration is not more common than it is. The answer lies in the remarkable ability of the gastric mucosa to defend itself. An ulcer may be considered to be the result of an imbalance between the aggressive contents of the stomach and this defence ability, and may result from an increase in the former or a decrease in the latter.

Until recently most studies on the pathogenesis of gastric ulceration and attempts at treating the lesion have been concerned with the roles of acid and pepsin and antagonism of their secretion or action. Little attention was focused on the role of the 'mucosal barrier' in ulcer formation or treatment of the disease by its stimulation. Indeed as recently as 1973 a Medical Research Council specialist committee on gastroenterology stated that the role of mucus in peptic ulceration was worthy of research.

Mucous glycoprotein synthesis cannot be satisfactorily studied \textit{in vivo} because of the poorly understood and probably complex control mechanism involved. The unsuitability of \textit{in vivo} studies was illustrated using the \textit{in vitro} assays based on the incorporation of radio-labelled precursors. Within an hour of an acute stress such as gastric intubation, glycoprotein synthesis was seen to decrease and did not return to normal levels until four hours. Such an effect of stress is certainly not limited to the stomach and indicates the need for care in the handling of animals for use not only in studies on glycoprotein synthesis but in all biochemical work.
The three anatomical divisions of the human stomach were seen to incorporate N-acetylglucosamine at different rates. Although it is possible that the observed differences may indicate the synthesis of glycoproteins with different carbohydrate side chains by the three areas it is more probable that the difference is due to the distribution of mucus secreting cells throughout the stomach, the pyloric antrum having the highest concentration. The inverse correlation between the rate of mucus synthesis and the tendency towards ulceration in a particular anatomical subdivision lends support to the protective role of gastric mucus with regard to ulceration.

That a derangement of mucus synthesis is involved in gastric disease is shown by the studies on biopsy samples from diseased human stomachs. Incorporation of hexosamine is decreased in those areas of the stomach affected by the lesion but is normal throughout the rest of the stomach. Thus decreased rates of mucus synthesis were seen in the lesser curve of patients suffering from ulceration of the lesser curve and in the antrum of gastritic patients. Similarly, malignant changes of the gastric mucosa were associated with decreased hexosamine uptake.

Carbenoxolone which has been shown in a large number of clinical trials (Pinder et al, 1976) to be efficacious in the treatment of gastric ulceration was shown to markedly increase mucus synthesis, measured by the incorporation of N-acetylglucosamine, to normal or greater levels during the course of successful therapy. This finding is in good agreement with that of Domschke et al (1972) who showed that N-acetylenuraminic acid levels which were depleted in the mucus of gastric ulcer patients were elevated by carbenoxolone treatment.
Contrary to the views of Lambert and Andre (1972) it was shown that the human gastric mucosa was capable of synthesizing sulphated glycoproteins. However, unlike the rat (Lambert et al., 1969) and the pig (Rainsford, 1975) in which ulcerogenic stimuli resulted in decreased sulphation of glycoproteins, sulphate incorporation was elevated in human chronic gastric ulcer and carcinoma. This may represent a species difference between humans and other species with regard to sulphation of glycoproteins. In rats carbenoxolone pretreatment resulted in increased synthesis of these compounds. It would be of interest to study the effects of the drug on sulphate incorporation in humans to establish whether the proposed species difference would result in carbenoxolone causing a decrease.

The majority of animal experiments concerning the ulcerogenic and anti-ulcerogenic effects of drugs on glycoprotein synthesis were carried out in the rat. Studies indicated that this species synthesised activated precursors and incorporated them into glycoproteins by established mechanisms. The water soluble mucosubstance consisted of low and high molecular weight glycoproteins (approx. $1.2 \times 10^5$ and in excess of $1 \times 10^6$ respectively) which appear to be related biosynthetically. It is possible that the higher molecular weight molecule may represent a repeating unit of the smaller glycoprotein. Similar results have been obtained by Allen and Snary (1972) for pig gastric mucin and by Lukie and Forstner (1972) for rat intestinal mucin. Thus the association of relatively low molecular weight subunits to produce large and complex glycoproteins may be general.

In general, factors which have been reported as causing mucosal damage led to a decrease in glycoprotein synthesis and those associated
with ulcer healing led to an increase when administered to rats. Thus the incorporation assay may represent a useful tool in screening new drugs for ulcerogenic or anti-ulcerogenic potential. However, the result with indomethacin and similar results which have been obtained for phenylbutazone (Ioannides, 1976) indicate that the length of the dosing period is critical since in the short term mucosal damaging agents have an apparent stimulating effect on glycoprotein synthesis, perhaps through an irritant effect on undamaged mucosal cells.

A number of mechanisms have been proposed for stress and drug induced gastric lesions including mucosal ischaemia resulting in impaired energy metabolism, damage to the endoplasmic reticulum, lysis of lysosomes and inhibition of hexosamine synthesis. All of these mechanisms will result (either as a primary effect e.g. direct enzyme inhibition, or a secondary effect, e.g. impaired energy metabolism) in interference with the synthesis of nucleotide sugars and a derangement of mucus synthesis which may aid the development of the observed lesion. In the case of ethanol and penicillin it may be argued that a disturbance in mucus synthesis precedes the development of the lesion since decreased incorporation rates were detected in mucosae without erosions as well as those with erosions.

In contrast to these agents carbenoxolone caused an increase in hexosamine incorporation when administered to rats. Although the rat is not the best experimental animal for use in studies on carbenoxolone since it has been shown to metabolise the drug somewhat differently to man (Iveson et al, 1971), carbenoxolone was shown to elicit the same effects on mucus synthesis in the stomach of the ferret which has been shown by Shillingford (1975) to metabolise the drug in the same way as man.
The effect of carbenoxolone on mucus synthesis appears to be on the structure of the oligosaccharide chain rather than on the overall amount of mucus synthesised, giving rise to a glycoprotein apparently richer in N-acetylhexosamines and sialic acid and depleted with respect to galactose. Menguy and Desbaillets (1968) found that mucin from rats treated with indomethacin and phenylbutazone which caused reductions in the concentration of protein-bound carbohydrates, particularly sialic acid and hexosamine, was digested more rapidly by pepsin than normal mucin. Thus by causing an increase in the concentration of hexosamines and sialic acids carbenoxolone will give rise to a glycoprotein which is more resistant to proteolytic digestion and a more protective mucosal barrier. Such an effect might explain the early observations of Hausmann and Tarnoky (1966) who found apparent increases in the amount of visible mucus in the stomachs of patients treated with carbenoxolone since a more stable mucus will persist for longer periods on the surface of the mucosa.

Carbenoxolone may also exert its beneficial action in the treatment of gastric ulceration through its ability to stabilize lysosomes. These organelles contain hydrolytic enzymes capable of degrading all cellular macromolecules and have been shown to release their contents under the influence of ulcerogenic stimuli such as stress (Fergusson et al., 1972) and non-steroidal anti-inflammatory drugs (Lewis et al., 1971). Among the macromolecules degradable by lysosomal hydrolases are the mucous glycoproteins (Chapter 4). Whether lysosomal rupture is a primary causative agent or secondary to the development of the ulcer the release of the contained lysosomes would certainly aid the further development of the lesion.

The stabilisation of lysosomal membranes although possibly of
importance in the ulcer healing action of carbenoxolone may be merely an example of a general effect of the drug on biological membranes. Such a general membrane action has been suggested by Symons (1976) and may explain other observed effects of carbenoxolone such as the decreased cell turnover noted by Lipkin (1971).

Similarly, the membrane activity of the drug may explain the observed effects on mucus synthesis. The build up of glycoproteins commences by the synthesis of the polypeptide chain in the ribosomes attached to the endoplasmic reticulum and proceeds by stepwise addition of carbohydrate units by specific glycosyl transferases which are also membrane bound. Thus it is conceivable that carbenoxolone may similarly stabilize the rough endoplasmic reticulum of the mucosal cell thus resulting in the observed changes in glycoprotein synthesis.

It may also be argued that gastric ulceration may arise through a derangement of the endoplasmic reticulum leading to production of a mucous glycoprotein with altered oligosaccharide chains giving a less protective mucus and an impaired mucosal barrier, resulting in the clinically observed lesion. This alteration in glycoprotein synthesis may be similar to the 'switchover' from the synthesis of secreted to non-secreted proteins associated with dissociation of the ribosomes from the endoplasmic reticulum. This process, which is normally regulated by steroid hormones (Sunshine et al, 1971) may explain the low incidence of gastric ulceration observed during pregnancy. Dissociation of the ribosomes is also stimulated by starvation and this has been shown to adversely affect mucus synthesis.

A similar degranulation i.e., the conversion of rough to smooth endoplasmic reticulum is caused by carcinogens. In this case, however,
the change is thought to be irreversible. A possible correlation between gastric carcinoma and ulceration has previously been mentioned and it has been shown that the effect on glycoprotein synthesis measured by the incorporation of hexosamine and sulphate due to the two lesions is similar. Furthermore, it has recently been shown that pretreatment of rats with carbenoxolone at dose levels which elicit effects on glycoprotein synthesis prevented the activation of biphenyl-2-hydroxylase, an indicator of ribosome removal and endoplasmic reticular damage, in the liver and stomach (Parke, 1976).

Schrager and Oates (1973) have shown that gastric carcinoma is associated with alterations in the structure of the glycoprotein oligosaccharides. It would be of interest to study hydrolysis fragments of glycoproteins from the mucus of gastric ulcer patients by the same technique of gas liquid chromatography to see if similar modified glycoproteins are produced in this lesion and whether carbenoxolone has a 'normalising' effect on this structure. Furthermore, the effect of carbenoxolone on the interactions of ribosomes with the endoplasmic reticulum with relation to ulcerogenesis and carcinogenesis warrants investigation.

The E type prostaglandins and their analogues also caused an increase in incorporation of hexosamine indicating that they too may exert their beneficial effects in the treatment of gastric ulcer by an effect on mucus synthesis. Moreover, the existence of endogenous E type prostaglandins in the stomach (Shaw and Ramwell, 1968) suggests that they may have a physiological role in the maintenance of the gastric mucosal barrier.

A correlation appears to exist between the actions of the prostaglandins
and carbenoxolone. Both have been shown to effect mucus synthesis and stabilise lysosomal membranes. The link between the two compounds is further evidenced by the finding of Peskar et al (1975) that carbenoxolone inhibited the enzymes responsible for the degradation of the prostaglandins. Hence the possibility that carbenoxolone might exert some of its beneficial action through potentiation of endogenous prostaglandins merits investigation.
BIBLIOGRAPHY
Abraham, R., Fabian, R. J., Golberg, L. and Coulston, F. (1970) Gastroenterol. 67 1169-1181


Avery Jones, F. and Parke, D. V. (1975) (Eds.) Fourth Symposium on Carbenoxolone (Butterworths, London)

Avery Jones, F. and Sullivan, F. M. (1972) (Eds.) Carbenoxolone in Gastroenterology (Butterworths, London)


Berstad, A. (1972) Scand. J. Gastroenterol. 7 129-135


Collin-Jones, D. E. and Taylor, J. V. Gut 14 423


Croft, D. N. (1973) Rendi di Gastroenterol. 5 73-79


Davenport, H. W. (1964) Gastroenterol. 46 245-253


Davenport, H. W. (1968) Gastroenterol. 54 175-181


Dean, R. T. (1975) Nat. 257 414-416


Desbaillets, L. and Menguy, R. (1967) Am. J. Dig. Dis. 12 582-588


Domschke, W., Domschke, S., Classen, M. and Demling, L. (1972a) Scand. J. Gastroenterol. 7 647-651
Domschke, W., Domschke, S., Classen, M. and Demling, L. (1972b) Acta Hepato. Gastroenterol. 19 204-205

Domschke, W., Domschke, S., Classen, M. and Demling, L. (1973) Nat. 241 454-455


Dragstedt, L. R. (1956) Gastroenterol. 30 208-214

Du Plessis, D. J. (1965) Lancet 1 974-978

Eastwood, G. L. and Kirchner, J. P. (1974) Gastroenterol. 67 71-84


Gallagher, J. (1976) Personal Communication


Huggins, C. and Talahay, P. (1945) J. Biol. Chem. 159 399-410

Ioannides, C. (1976) Personal Communication

Iveson, P., Lindup, W. E., Parke, D. V. and Williams, R. T. (1971) Xenobiotica 1 79-95

Ivey, K. J. (1971) Gastroenterol. 61 247-256

Ivey, K. J. (1972) Gut 12 750-757


Ivey, K. J. and Gray, C. (1973b) Gastroenterol. 64 1101-1105


Mao, C. C., Shanbour, L. L. and Hodgins, D. S. (1973) Gastroenterol. 63 427-438


Martin, F., Vuez, J., Berard, A., Andre, C. and Lambert, R. (1968) Dig. 1 165-174


Menguy, R. and Desbaillets, L. (1967b) Am. J. Dig. Dis. 12 862-866


Parke, D. V. (1976) Personal Communication


Peskar, B. M. (1975) Wein. Z. Inn. Med. 54 312-313


Puuren, J. and Karppanen, H. O. (1975) Life Sci. 16 1513-1520

Rainsford, K. D. (1975) Agents and Actions 5 326-344

Rhodes, J. (1972) Gastroenterol. 63 170-182


Robson, J. M. and Sullivan, F. M. (Eds.) A Symposium on Carbenoxolone (Butterworths, London)


Salganick, R. I., Argutinskaya, S. V. and Bersimbaev, R. I. (1972) Experientia 28 1190-1191


Schrager, J. (1969) Digestion 2 73-89


Schrager, J. and Oates, M.D.G. (1971) Digestion 4 1-12


Segal, H. L. (1975) In Dingle, T. J. and Dean, R. T. (Eds.) Lysosomes in Biology and Pathology Vol. 4 p. 295-302 (N. Holland Publishing Co.)


Watkinson, G. (1960) Gut 1 14-30


Slomiany, B. L. and Meyer, K. (1972) J. Biol. Chem. 247 5062-5070

Slomiany, B. L. and Meyer, K. (1973) J. Biol. Chem. 248 2290-2295


Sullivan, F. M. (1972) In Avery Jones, F. and Sullivan, F. M. (Eds.) Carbenoxolone in Gastroenterology p. 3-18 (Butterworths, London)


