PHYSICAL AND CHEMICAL CHANGES IN PORCINE GASTRIC MUCUS IN THE NORMAL AND ULCERATED STATES

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ERRATUM IMPAGINATION

Page 116

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TO

SHAIDA AND SUMAYA

For being so patient and understanding throughout.

ABSTRACT

It has been reported that there is a 100% incidence of ulceration of the squamous pars oesophagea of the pig's stomach following bile duct ligation. The reproducibility of this model has made possible its use in the investigation of the biochemical aspects of mucus secretion in both the normal state and at various states of ulceration.

The main findings are summarised below:

Mucus scrapings of the cardiac gland region of the pig stomach had a higher water and total protein content in the pre-ulcerated, ulcerated and post-ulcerated states. Gel chromatography on Sepharose CL-4B indicated larger amounts of degraded mucins relative to native mucins in the samples obtained from pre-ulcerated, ulcerated and post-ulcerated stomachs, as compared with the normal and control samples. The amounts of purified mucins obtained after isopycnic centrifugation in CsCl and gel chromatography on Sepharose CL-2B decreased from the normals and controls to the bile duct-ligated pigs.

An analysis on SDS-PAGE revealed a considerable degree of proteolytic degradation of the pig gastric mucins in the bile duct-ligated pigs as compared with the normal and control animals. Staining reagents specific for both the protein and the carbohydrate components of the mucus glycoprotein were employed in gel electrophoresis, which also revealed the presence of contaminating protein, viz. haemoglobin, pepsin, albumin and smaller glycoproteins, to a greater extent in the ulcerated than in the normal and control states.

Since pig gastric mucins contain 75% of carbohydrate, the determination of the proportions of monosaccharide constituents was essential. This was performed by GLC analysis of the alditol accetate derivatives of the sugars, which were characterised by mass spectrometry as well as by their retention times, relative to standards, on both packed and capillary columns. The most striking changes indicated by the GLC analyses were a decrease in the fucose content of the mucins from the normal to the ulcerated states and an increase in the proportion of N-acetylglucosamine in mucins from sham-operated animals.

The control (sham-operated) pig behaved very similarly to the normal pig for up to 24 hours after the surgical procedure. At 48 hours, however, slight changes resembling those following bile duct ligation occurred. This could be due to the trauma of the sham-operation, which involved the surgical insertion of a cannula in the body region of the pig's stomach. It is possible that, under such stress biosynthesis of the mucins may be affected.

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GLOSSARY

A	Antrum (antral gland area) of pig stomach.
B	Body region of pig stomach
BCB	Brilliant coomassie blue
BSA	Bovine serum albumin
C	Cardiac gland area of pig stomach
CsBr	Caesium bromide
$CHCl_3$	Chloroform
CsCl	Caesium chloride
DGU	Density gradient ultracentrifugation
EtOH	Ethanol
g	gram
GLC	Gas liquid chromatography
HPLC	High performance liquid chromatography
HSA	Human serum albumin
I.D.	Internal diameter
M	Molar
M/e	Charge to mass ratio
Mass spec	Mass spectrometry
MW	Molecular weight
N	normal pig
NaCl	Sodium chloride
nm	Nanometre
P	Pars oesophagea

PAGE	Polyacrylamide gel electrophoresis
PAS	Periodic acid Schiff's reagent
POU	Post-ulcerated
PU	Pre-ulcerated
RIA	Radioimmunoassay
std	Standard
S.D.	Standard deviation
S.D.S.	Sodium dodecyl sulphate
s.m.	Starting material
U	Ulcerated pig
Vi	Included peak
V_o	Void volume
ww	wet weight
w	weight
24 hr.C	Twenty-four hour control
48 hr.C	Forty-eight hour control

INDEX

Page

TITLE.. i

DEDICATION... ii

ABSTRACT... iii

ACKNOWLEDGMENTS.. v

GLOSSARY... vii

INDEX.. ix

FIGURES................ xiii

PLATES... xv

TABLES... xviii

APPENDIX... xix

1 OVERVIEW OF GASTRIC MUCUS AND ULCERATION............... 1

1.1. Nomenclature of mucus.......................... 1

1.2 Body's defence against gastric ulceration....... 5

1.3 An overview of mucus............................ 9

1.4 Structure of mucus.............................. 18

1.4.1 The subunit............................ 18

1.4.2 The protein backbone................... 19

1.4.3 The carbohydrate side-chains........... 21

1.4.4 Structure and physiology of gastro-intestinal mucus........................ 23

1.4.5 The structure,viscosity and gel-forming properties of mucus............ 24

1.5 A hypothesis for protection.................... 26

1.5.1 Bicarbonate ions........................ 28

1.6 Mucus from other sources in the gastro-intestinal tract of the pig.................... 29

1.7 The biosynthesis, regulation and mechanism of secretion of gastrointestinal mucus.......... 30

1.8 Histology of the pig stomach.................... 36

1.8.1 Introduction........................... 36

1.8.2 The pig as an experimental model in gastric ulceration.................. 36

1.8.3 The different anatomical areas of the pig stomach....................... 39

1.8.4 Mucus production in different areas of the porcine stomach................ 43

1.9 Definition of an ulcer......................... 45

1.9.1 What is gastric ulceration?............ 45

1.9.2 A general definition of an ulcer....... 46

1.9.3 Definition of an ulcer as applied to the stomach........................... 46

1.9.4 Usage of the term "peptic ulcer"....... 47

1.10 Mucus in disease.............................. 48

1.11 Drug effects on mucus and its secretion......... 57

2. EXTRACTION AND PURIFICATION OF PIG GASTRIC MUCUS....... 68

2.1 Introduction.................................. 68

The pig as a model for the study of mucus in mucus in gastric ulceration..................... 68

2.1.1 Clinical significance of the study..... 69

2.1.2 The mucus scrapings.................. 69

2.2 Methods and Materials.......................... 69

2.2.1 Source of pigs........................ 69

2.2.2 Pre-operative management............... 70

2.2.3 Anaesthesia........................... 70

2.2.4 Operative technique................... 70

2.2.5 Ligation and division of the common bile duct............................ 71

2.2.6 Insertion of gastric cannula........... 71

2.2.7 Control (sham) operation............... 72

2.2.8 Post-operative management and endoscopic assessment..................... 72

2.2.9 Removal of stomach.................... 72

2.2.10 Preparation of the stomach............ 73

2.2.11 Parameters for estimation............. 73

2.2.12 Measurement of surface area........... 75

2.2.13 Extraction and purification of mucus... 75

2.3 Results and Discussion......................... 77

2.3.1 Endoscopy and histology of normal and ulcerated stomachs.................... 77

2.3.2 Amounts of water in scraped material from normal and operated pigs.......... 79

2.3.3 Protein concentration under different conditions............................ 79

2.3.4 Purification and estimation of gastric mucins.................................. 80
2.3.5 Discussion.................................. 83
2.4 Summary and discussion.................................. 84

3. CHARACTERISATION OF PIG GASTRIC MUCUS BY ELECTROPHORESIS.................................. 117
3.1 Introduction.................................. 117
3.2 Materials and Methods.................................. 119
3.3 Results.................................. 122
3.4 Discussion.................................. 128

4. CARBOHYDRATE ANALYSIS OF PORCINE GASTRIC MUCIN FROM NORMAL AND ULCERATED ANIMALS.................................. 143
4.1 Introduction.................................. 143
4.2 Materials and Methods.................................. 144
4.2.1 Hydrolysis and derivatisation of mucin and carbohydrate chains........... 144
4.2.2 Analysis of alditol acetates by GLC..... 145
4.3 Results and Discussion.................................. 146

5. CONCLUSION.................................. 150

REFERENCES.................................. 154

APPENDIX.................................. 168

LIST OF FIGURES

		Page
Figure 1	The mucus glycoprotein basic unit............	17
Figure 2	The carbohydrate side-chains in pig and human gastric mucin..............................	20
Figure 3	A proposed model for the two-component defence mechanism.................................	27
Figure 4	Purification of pig gastric mucus glycoprotein on Sepharose CL-4B, read at an absorbance of 280 nm......................................	86a
Figure 5	Centrifugation of pig gastric glycoprotein (from peak A, Fig.4) on CsCl density gradient.	87
Figure 6	Further purification of peak B (Fig.4) on Sephacryl S-300............................	88
Figure 7	Purification of pig gastric mucins for PAS positive fractions (Fig. 5) on Sepharose CL-2B.......................................	89
Figure 8	The water content of gastric mucus scrapings from pigs of various conditions..............	90
Figure 9	The protein content of gastric mucus scrapings from pigs of various conditions..............	91
Figure 10	The degradedglycoprotein to protein ratio of fractions pooled from peak B (Fig.4) in pigs of various conditions........................	92

Figure 11	The degraded glycoprotein to protein ratio of fractions pooled from peak B (Fig.4) in pigs of various conditions	93
Figure 12	The amounts of pure pig gastric mucins in the various conditions	94
Figure 13	The amounts of pure pig gastric mucins in the various conditions	95
Figure 14	GLC profile of derivatised alditol acetates	148a
Figure 15	GLC profile of derived alditol acetates from a normal pig	148b
Figure 16	GLC profile of derived alditol acetates from 24 hour control pig	148c
Figure 17	GLC profile of derived alditol acetates from a 48 hour control pig	148d
Figure 18	GLC profile of derived alditol acetates from a pre-ulcerated pig	148e
Figure 19	GLC profile of derived alditol acetates from an ulcerated pig	148f

LIST OF PLATES

Plate 1 Polymeric structure of pig gastric mucin....... 22

Plate 2 The pig stomach (normal) opened along the greater curvature.......................... 40

Plate 3 The pig stomach (ulcerated) opened along the greater curvature.......................... 41

Plate 4 The constituents of pig gatric mucus.......... 96

Plate 5 A pig slung on a Pavlov frame during endoscopy. 97

Plate 6 The normal intact pars oesophagea as seen through the endoscope........................ 98

Plate 7 The first visible sign of changes (arrow) as seen through the endoscope, 16 hours after the operation, the early pre-ulcer stage........... 99

Plate 8 Denudation of portions of the pars (arrow) as seen through the endoscope at 19 hours after the operation; the early pre-ulcer stage....... 100

Plate 9 Inflammation and signs of bleeding (arrow) of the pars 21 hours after the operation; the late pre-ulcer stage..................... 101

Plate 10 Bleeding and complete denudation of the pars at 24 hours; the ulcer stage................. 102

Plate 11 A macroscopic view of a normal pig stomach showing intact, bile stained pars (arrow)...... 103

Plate 12 A macroscopic view of an ulcerated pig stomach with denuded pars (arrow) and evidence of bleeding.. 104

Plate 13 A histological section of the normal pars-cardia junction of the pig stomach............ 105

Plate 14 A histological section of the normal pars-cardia junction of the pig stomach stained differently (see text)...................... 106

Plate 15 A histological section of the pars of normal pig stomach.................................. 107

Plate 16 A histological section of the cardiac gland area in a normal pig stomach................. 108

Plate 17 A histological section of the considerably denuded pars of a pig stomach during the stage of pre-ulceration........................ 109

Plate 18 A histological section showing the margin of the ulcer (arrow) in the pars region of an ulcerated pig stomach......................... 110

Plate 19 A histological section of an eroded pars (arrow) in an ulcerated pig stomach showing the complete loss of surface epithelium................... 111

Plate 20 A histological section of the ulcerated pars cardia junction showing a typical cuff of stratified squamous epithelium (arrow) at the distal margin of the ulcer............... 112

Plate 21	0.1% SDS 10% PAGE	132
Plate 22	0.1% SDS 4% to 20% gradient PAGE	133
Plate 23	0.1% SDS - 10% PAGE	134
Plate 24	0.1% SDS - 4% to 20% gradient PAGE	135
Plate 25	0.1% SDS 4% to 20% gradient PAGE	136
Plate 26	0.1% SDS-PAGE stained with BCB	137
Plate 27	0.1% SDS-PAGE stained with $AgNO_3$	138
Plate 28	0.1% SDS - 4% to 20% gradient PAGE; stained with NH_3AgNO_3	139
Plate 29	0.1% SDS - 4% to 20% gradient PAGE stained with NH_3-$AgNO_3$	140
Plate 30	0.1% SDS - 4% to 20% gradient PAGE stained with NH_3-$AgNO_3$	141
Plate 31	0.1% SDS - 4% to 20% gradient PAGE stained with NH_3-$AgNO_3$	142

LIST OF TABLES

Table 1 Differences between glycoproteins and proteoglycans.............................. 2

Table 2 Properties of mucus glycoproteins compared with those of plasma glycoproteins........... 4

Table 3 Values of total protein per wet weight and dry weights per cm^2 of normal, pre-ulcerated, ulcerated and post-ulcerated pigs............. 113

Table 4 Carbohydrate to protein ratio in Sepharose CL-4B included peak.......................... 114

Table 5 Values of mucin concentration per weights and volume per cm^2 of normal control, pre-ulcerated, ulcerated and post-ulcerated pigs.. 115

Table 6 Ratios as determined by GLC of the constituent monosaccharides of pig gastric mucins, from the normal to the post-ulcerated states (as determined on an OV-225 packed column)............ 149

APPENDIX

Appendix A	Scrape of mucus.........................	168
Appemdix B	The Bio-Rad Assay........................	169
	The Bio-Rad standard curve................	170
Appendix C	The PAS standard curve...................	171/172
Appendix D	Staining procedure	173
Appendix E	The $AgNO_3$-NH_3gel development system.......	174
Appendix F	Peracetylated N-acetylhexaminitol.........	175
Appendix G	6-Deoxyhexitol penta-acetate (fucose alditol acetate)........................	176
Appendix H	Hexitol hexa-acetate (Galactose alditol acetate)................................	177
Appendix I	Peracetylated N-acetylhexosaminitol (Gal Nac and GlcNAc).......................	178
Appendix J	Hexitol hexa-acetate (GAL alditol acetate).	179
Appendix K	Normal mucin............................	180

1. OVERVIEW OF GASTRIC MUCUS AND ULCERATION.

The aim of this study was to investigate both quantitative and qualitative changes in the composition of pig gastric mucus in the normal and ulcerated states.

Gastric ulceration has been the focus of attention for many years. Workers in this field of study have sought reasons for the ability of the stomach to withstand the corrosive nature of its gastric juices. The concept of a 'gastric mucosal barrier', although not too clearly defined in the early years, has evolved from the initial idea of a single mucosal epithelial barrier to a two component epithelial barrier. Mucus, a thick, viscid, semi gel substance is regarded as one component of this barrier. The other component is thought to be a layer of HCO_3^- in between the mucus layer and the mucosal epithelium. Although changes in both the concentration and composition of the mucus are evident between the normal and ulcerated states, it is still a matter of speculation whether these changes are a cause or a consequence of the process of ulceration.

1.1. Nomenclature of mucus

Mucus is widespread in nature, being present in many species ranging from the earthworm to man, and has evolved to fulfil a number of extracellular roles. In humans, mucus is of obvious importance in systems as functionally different as the respiratory, gastrointestinal and genitourinary tracts. In addition to the medical interest, the fascinating chemical and physical properties of mucus have long been an object of interest to biochemists and physical chemists. It is not surprising, therefore, that a subject studied by so many different disciplines should

TABLE I

Differences between glycoproteins and proteoglycans

Characteristic	Glycoprotein	Proteoglycan
Principal sites in the body	Membranes; body fluids & secretions (e.g. blood, mucus)	Skeletal & supporting tissues (e.g. cartilage, bone)
Carbohydrate component	Oligosaccharide unit	Glycosaminoglycan
Size of carbohydrate component	Less than 25 monosaccharides	More than 50 monosaccharides
Linkage monosaccharide	N-acetylhexosamine	Xylose
Repeating structure	Little or none	Repeating disaccharide
Shape of carbohydrate component	Branched	Linear, unbranched
Hexuronic acid	Absent	Present

The above characteristics are typical of most glycoproteins and proteoglycans but, as always in biological substances, there are exceptions. For example, keratan sulphate does not contain hexuronic acid.

have led to so much confusion in the nomenclature both of mucus and related materials (Clamp, 1980).

To quote an example of the confusion that has prevailed in the nomenclature, the term "mucoid" has been defined in at least four different ways:

Clinicians have used "mucoid" to mean a non-purulent sputum; biochemists have defined "mucoid" as any glycoprotein with a hexosamine content greater than 4%, histochemists apply the term to any neutral "mucin"; gynaecologists use "mucoid" for a type of cervical mucus (Reid et al., 1978).

The confusion exists for a host of other terms such as "mucoprotein", "mucosubstance", "mucopolysaccharide", etc. To overcome the many problems, it has been decided to abandon imprecise terms such as "mucoid" (Reid et al., 1978, Clamp et al., 1980) or those prefixed by "muco-", together with many old and rigid classification systems. The total secretion is "mucus". The major component of mucus that confers the characteristic physicochemical properties upon the secretion is widely referred to as "mucus glycoprotein". The degradation products of the glycoprotein are "glycopeptides". The term "glycoconjugates" embraces all those polymeric materials containing covalently linked carbohydrate, but excluding nucleic acids. "Glycoconjugates" may refer to molecules with carbohydrate linked to either lipid or protein. There are two quite different types of glycoconjugate in which carbohydrate is linked to protein, namely the proteoglycans and the glycoproteins. Table I gives the essential differences between the two types of glycoconjugates.

"Mucus glycoproteins" could be a rather cumbersome term to use,

TABLE 2

Properties of mucus glycoproteins compared with those of plasma glycoproteins

Characteristic	Mucus glycoprotein	Plasma glycoprotein
Amino acid content	High levels of serine, threonine & proline. Low levels of aromatic & sulphur-containing amino acids	Amino acid spectrum of a typical protein
Carbohydrate content	More than 50%	Less than 25%
Linkage	N-acetylgalactosamine to serine or threonine (O-glycosidic)	N-acetylglucosamine to asparagine (N-glycosidic)
Monosaccharides Fucose Galactose N-acetylglucosamine Sialic acid	Present	Present
Mannose	Low levels or absent	Present
N-acetylgalacto-samine	Present	Low levels of absent

There are several different types of glycoprotein but, for the purposes of this Table, mucus glycoproteins are compared with plasma glycoproteins. There are exceptions to the above statements: for example, IgA1, a plasma glycoprotein, contains O-glycosidically linked units.

especially if it is frequently required. Also, besides being found in the major secretions of the body, glycoproteins also occur in plasma. The immediate difference between these types of glycoproteins are their sizes and the amount of carbohydrate associated with each type. Usually plasma glycoproteins have a carbohydrate moiety of less than 4% whilst "mucus glycoproteins", whose molecular weights could range from one to fifteen million have anything between 50 and 90% carbohydrate. Table II provides some of the more essential differences.

Therefore it has become common practice to refer to "mucus glycoproteins" with large amounts of carbohydrate associated with them as "mucins", along with their specific locations so that we have gastrointestinal mucins or cervical mucins etc. Further descriptive terms may be applied eg. neutral, acidic, sialyated, sulphated etc.

1.2. Body's defence against gastric ulceration

Although it has long been recognised that gastric mucosa is capable of resisting acid and peptic digestion, adequate explanations of the mechanism responsible for this eluded workers until recent times. An increased secretion of acid provides only a partial explanation, at best, for peptic ulceration. "No acid, no ulcer", a famous dictum enunciated by Schwarz in 1925 (Grossman, 1979). This was regarded as being essentially correct until it became clear that although this might be the case for duodenal ulceration, which is characterized by a high secretion of acid, gastric ulcers, in contrast, may occur in persons who have barely detectable levels of acid secretion (Davenport, 1965). These observations shifted the focus of attention to the possibility of the existence of mucosal components that

might play a role in preventing damage. From this arose the concept of the "gastric mucosal barrier", the nature of which proved a problem to elucidate. For some time differing views were held by various workers on the nature of this barrier until the last few years when these viewpoints started to converge on the important role of mucus in providing an unstirred layer on the mucosal surface on which a layer of bicarbonate ions is maintained (Rees et al., 1982). Mechanisms of gastric mucosal protection are now explained in terms of the mucus-bicarbonate barrier.

The notion that the layer of mucus that covers the gastric mucosal surface protects it against the injurious action of the acid is so old that its origins cannot be accurately identified (Grossman 1979). For example, Claude Bernard in 1855 wrote that the mucus lining the stomach encloses the gastric juice in a vase as impermeable as though it were porcelain (Davenport, 1972). Bernard also held the view that gastric mucus impedes the movement of luminal pepsin to mucosa (Bickel et al., 1981). The same author refers to Pavlov as saying in 1910 that gastric surface mucus is important by virtue of its flowing action which washes away pepsin and other noxious substances. It is therefore somewhat surprising that Davenport (1964), proposed that the barrier to ionic diffusion resided within the surface epithelium, probably in the apical membrane and tight junctions between cells. Damaging agents, such as aspirin and bile salts, were seen to disrupt this barrier resulting in the subsequent increase in acid back-diffusion leading to the release of histamine and other mediators of tissue inflammation, thus initiating the process leading to mucosal damage. (Davenport et al., 1964, 1967). Modern textbooks quote this mechanism widely. However, doubt has been cast on its applicability

to all types of mucosal damage.

The hypothesis of the "back diffusion of acid" (Thjodleifsson et al., 1977) dominated the literature for many years. It was tested by Teorell, about forty years ago, (Teorell, 1939, 1940) who considered that protons underwent exchange-diffusion with sodium ions, the latter appearing in the gastric lumen in place of the hydrogen ions. Gastric mucosa was exposed to bulk solutions containing known amounts of acid and the residue withdrawn after intervals of time to increase the recovery of instilled acid. This concept was given an enormous boost when it was reported that when certain chemical and physical agents such as carboxylic acids, in particular acetylsalicylic acid, were applied to the luminal surface of the gastric mucosa, exogenous acid disappeared at a greatly increased rate. In other words, there was more "back-diffusion" of acid because the normal "barrier" to back-diffusion of acid had been destroyed by the agents mentioned and the mucosa had, therefore, been rendered permeable. The hypothesis was extended to explain the low acid concentration of the juice secreted by chemically treated and injured mucosa, since it had been observed that patients with gastric ulcer secrete juice which contains abnormally little acid but high concentrations of sodium ions (Teorell, 1939). However, these theories have incurred harsh criticism in recent times (Thjodleifsson et al., 1977) and it has been stated that the hypothesis and its underlying assumptions lack direct evidence. By this time alternative explanations for mucosal protection had appeared in the literature, and the role of mucus was seen to be of prime importance (Allen et al., 1972, Allen, 1978).

The "barrier" role of the extracellular layer of mucus had received attention from time to time since the 1800's. In his paper on "The two

component mucous barrier", Franklin Hollander (1954) briefly traces the historical opinion on mucus. In 1800, Glover, in a doctoral dissertation, wrote concerning the mucus secretion of the digestive tract: "Its use seems to be that of lubricating ... It must likewise defend the internal surface of the stomach and intestines, from the action of the gastric juice, and from the acritude of bile when regurgitated." Sixty years later, Harley, enforced this view by a statement, "it is chiefly, if not solely, the mucus which protects the stomach from the chemical action of its own gastric juice."

However, the view that mucus acted as a physical barrier fell into disfavour when it was argued that it consists of over 95% water and could hardly be expected to delay the diffusion of hydrogen ions across it. It was thought that mucus provided merely an unstirred layer of water on the epithelium when it was demonstrated that the diffusion of hydrogen ions through mucus was similar to that of water (Heatley, 1959). However, recent observations have indicated that the rate of transfer of hydrogen and sodium ions through mucus is delayed to a greater extent than the rate of diffusion through unstirred water (Williams et al., 1980). Hydrogen ions traversed mucus at a rate three to four times slower than that through a similar thickness of unstirred water. The discrepancy between these results and Heatley's may be resolved by noting that the earlier experiments were performed with dilute solutions of mucus whereas the recent tests were performed with concentrations similar to those in the mucus layer on the gastric mucosa (Rees et al., 1982). Anyway, interest in mucus was rekindled when Hollander noted its importance as one component

of a two- component barrier (Hollander 1954). Epithelial renewal was the proposed second barrier.

However, the consensus of opinion remained against the view that there was an extracellular component to mucosal protection. It was only with the recent development of techniques for measuring secretion of bicarbonate by gastric mucosa, that the importance of mucus as a surface epithelial unstirred zone, in which luminal acid and epithelial alkali may mix, has been recognized. These studies have allowed the concept of a "mucus-bicarbonate" barrier to be developed (Allen et al., 1980 and 1982; Allen, 1981; Rees et al., 1982). Further evidence supports this hypothesis with the demonstration of a pH gradient across the mucus layer adherent on the surface of the gastric mucosa in rabbits (Williams et al., 1981) and humans (Bahari et al., 1982).

1.3. An overview of mucus

Although it is has long been known that the gastric mucosa is capable of resisting peptic digestion, the actual mechanism and physiological basis of this resistance has not been adequately explained.

The early models which were formulated to elucidate how much protection was brought about, attached great importance to the apical membrane of surface epithelial cells and tight junctions linking adjacent cells (Davenport et al., 1964; Davenport, 1967). Mucus was vaguely considered to play either a lubricative or protective role or both (Heatley, 1959; Florey, 1962). During this early period, there was a brief flurry of interest in the study of gastric mucosal protection when it was proposed that mucus was one component of a two-component barrier system

(Hollander, 1954) with the subjacent layer of epithelial cells making up the second component.

Studies of mucus originally began with a close look at secretions in lower animals. More recently mucus of the mammalian gastrointestinal, genital and respiratory tracts have been studied. Mucus secretions in lower and higher forms of life are regarded to be of utmost importance since they play a significant role in a variety of functions. Sir Francis Avery Jones (1977), stated that "we cannot do without mucus, irritating though it may be if there is too much or too little, and in so many systems of the body it plays a vital role." Furthermore, he called mucus "one of nature's perfections in protection" (Jones, 1978). The need for a multidisciplinary approach to the study of mucus has been emphasized, because it is a unique biological material found throughout the animal kingdom at many levels of organisation (Elstein et al., 1977).

The notion that the layer of mucus covering the gastric mucosal surface protected it against the injurious action of stomach acid in some way was proposed by many early workers in the mucus field but they viewed _inter alia_ its main role as one of lubrication (Grossman, 1979 _inter alia_). Clearly, the stomach lining requires a substance or substances which can protect it from the forces of mechanical erosion and this mucus fitted into this role admirably. (Hafez, 1977; Silberberg et al., 1982).

The role of mucus in the survival of lower animals cannot be underestimated, and again lubrication appears to be its main function. However, mucus secretions associated with the external surfaces of lower animals act both as a lubricant and as a waterproofing substance protecting the organism from sudden changes in osmotic pressure (Negus, 1967; Jones,

1978). Mucus provides a permeable skin for the earthworm, (Lumbricus), slugs, (Limax), frogs, (Rana), aquatic toads (Xenopus) and fishes such as eels (Anguilla). The outer cover of mucus allows the passage of oxygen and carbon dioxide through the skin, which is essential in the species mentioned, since they breathe partly or entirely through their skins.

But it is the control of the passage of water through the skin that makes the mucus layer so important (Hafez, 1977). Eels, for example, have a skin made up of layers of goblet cells and, if their mucus covering is removed, they gain weight by osmosis when placed in distilled water and lose weight when placed in hypertonic saline. In some fish such as Carp, the olfactory recess is studded with goblet cells and the mucus helps to control the passage of water.

In higher animals, mucus plays important roles in many parts of the body. For instance, in the respiratory tract it plays a vital role, together with the cilia, in the protection of the sensitive epithelium and in the removal of foreign particles and inspired chemicals (Reid et al., 1982; Yeats, 1982). Mucus maintains an unbroken sheath over the entire respiratory surface and retains foreign particles brought into contact with it (Hafez, 1977). Here mucus is essential for the transport of dust, debris, irritants and bacteria from the lungs. Any foreign body that enters the airways is rendered less harmful by mucus. There is growing evidence that mucus has antiviral and antibacterial properties (Forstner, 1978; Gallagher et al., 1982). Mucus has been shown to contain immunoglobulins, mainly secretory IgA; lactoferrin which chelates iron necessary for the growth of some bacteria and lysozyme which destroys some bacteria, (Jones et al., 1978). The control of mucociliary function in the

respiratory tract is basic to the prevention and management of respiratory illness (Lopez-Vidriero et al., 1978; Reid et al., 1982).

In many cases mucins enhance host resistance to bacterial infection (Forstner, 1978). It was found that salivary mucin competes with receptor groups on buccal epithelium which are specific for some pathogenic streptococci. The mucin was able to bind the organisms into aggregates (Kashket et al., 1972), which could be swept away by saliva, thus preventing the bacteria from embedding in the buccal mucosa and setting up a focus of infection. Further evidence for the successful competition of mucin for epithelial receptors has been shown in the case of intestinal mucin and cholera toxin (Strombeck et al., 1974).

Pig gastric mucus can bind cholera toxin and prevent its attachment to the cell surface (Allen, 1981). Mucus may either provide a barrier for the pathogenic organism and thus protect the mucosa or alternatively combine directly with the organism or its toxin and thereby inactivate it. It has also been shown that crude gastric mucin enhances the growth of a porcine parasite, Balantidum coli, (Klass, 1974), which may infect the gastro-intestinal tract of humans and some experiments have suggested that mucins have the capacity to act as a culture medium for certain human pathogens. This may be seen as advantageous to the host, since as long as the mucin culture medium remains mobile, organisms will pass out with the faeces without invading the mucosa.

Mucus has been shown to be important for the maintenance of the gastro-intestinal flora: for example, salivary mucus interacts with oral streptococci (Gibbons et al., 1978; Levine et al., 1978), and soluble mucus is a nutrient source for endogenous bacteria in the gut (Hoskins, 1978).

Other functions of mucus include binding of cations such as iron and calcium (Forstner et al., 1975), the significance of which is not yet known. Di- and trivalent cations have been demonstrated to thicken mucus gels and the effect is concentration dependent. The changes produced have been shown to be due to conformational alterations of the glycoproteins in solution (Marriot, 1982). It is known that cation levels in human cervical mucus alter at various stages during the menstrual cycle and this may be part of the whole control mechanism in relation to fertility.

As already mentioned an important link in the hypothesis of mucus defence is the presence of a major subclass of IgA antibodies that have been found in mucus secretions (Tomasi, 1972). IgA, has been shown to have a non-immunoglobulin glycoprotein between its Fc and Fab subunits, called secretory component (Allen, 1981), of molecular weight 70,000. It has been shown to have a mucus like domain in its structure (Clamp, 1977, 1980) and presumably it would be miscible with mucus. The consequence of the structural features is that IgA is able to form a monolayer of antibodies at the mucus surface. The mucus-like portion of the molecule, with its surface specialisation, lies in the mucus phase, the rest of the molecule in the overlying fluid. This antibody monolayer on the surface of the mucus thus plays a vital role in the defence against pathogens.

The model described above is not the only one postulated and other types of interaction between mucus and IgA have been proposed. These other models however, have several disadvantages over the one discussed above: for example, if the IgA were not "hinged" on to the mucus via its secretory component but was secreted into the bulk of mucus, it would not have access to its antigen. Likewise, if it were secreted into the fluid overlying the

mucus e.g. into gastric juice, it would become too diluted to be effective.

Interestingly, IgA has a deletion of the heavy chain in which the mucus-like domain of IgA residues reside and therefore because of an inability to interact with the mucus layer only resides (Clamp, 1980),in the fluid phase which overlays the mucus. In this way it is regarded as a first line of defence.

In relation to this discussion about IgA mucus interaction playing a defensive role against pathogens it may be asked what sequence of events allow certain viruses to penetrate mucus successfully and attack underlying cells. Viruses may destroy or penetrate the mucus barrier by coating themselves with glycoproteins miscible with the mucus. Vibrio cholerae is thought to secrete neuraminidase which enables it to cleave off terminally located, negatively charged sialic acid residues and thus bore a channel through the mucus.

In the mammalian genital tract, mucus exerts a minor regulatory role in the passage of the sperm to the upper genital tract (Chantler, 1982). Semen is rich in blood group substance and therefore presumably in mucus type glycoprotein (Gibbons, 1978). The Cowper's glands secrete a mucin of molecular weight $6,5 \times 10^6$ (Boursnell et al.,1970) which forms a gel with other components secreted by the male accessory glands and disperses spontaneously under the influence of seminal proteolytic enzymes (Syner et al., 1972).

In the female the physicochemical properties of mucus show a cyclic variation during the menstrual cycle (van Kooij et al., 1980, 1982), and its variation during ovulation facilitates the transport of sperm (Edwards, 1978). Estimation of the time of ovulation may be made merely by

observation of the changes in the colour, consistency and amount of cervical mucus (Flynn et al., 1976). Cervical secretions play an important role in the protection of the sperm in the inhospitable environment of the vagina (Elstein, 1978; Nirmala et al., 1982). Most of the functions of the cervix are affected by the secretion of the cervical epithelium (Elstein, 1978, 1982).

Mucins are produced and secreted from epithelial cells throughout the gastrointestinal tract, including salivary glands, oesophagus, stomach, intestines and colon (Forstner, 1978). In the small intestine the mucus-containing goblet cells are interspersed in an organised fashion among absorptive cells, gradually increasing in number towards the large intestine. In the descending colon and rectum of humans, goblet cells are numerous, comprising one eighth of the epithelial cell population.

There are considerable variations in the structure of the carbohydrate chains in glycoproteins from different regions of the gastrointestinal tract in the same species as well as between species (Allen, 1981). However, mucus throughout the gastrointestinal tract is assumed to provide lubrication and protection of the underlying cells against potentially injurious chemicals, enzymes, bacteria and dietary constituents (Forstner, 1978).

To understand the physiological function of mucus secretions in general and especially cervical mucus in relation to sperm penetration, a model involving the hypothesis that mucus is a medium in which most macromolecules are "insoluble" has been proposed. Small molecules are soluble and free to diffuse through this mechanism and thus the prime role of mucus at the molecular level might be selectively to exclude macro-

molecules and larger particles from contact with cell membranes. Thus mucus could be described as a fluid molecular sieve allowing the penetration of only certain substances, the bulk of material coming into contact with it being screened out. At equilibrium, in gastric juice, for example, the concentration of macromolecules in the mucus could be orders of magnitude less than in total gastric juice, since they are unable to dissolve into the mucous gel. This is in contrast with an older idea (Schrager et al., 1970), that macromolecules were simply slower to diffuse into mucus.

This hypothesis is particularly attractive in the sense that specific macromolecules may have to have surface specialisations to be soluble in the mucus phase. This is a possible explanation for the protection of the upper genital tract from invasion by vaginal micro-organisms, and for explaining the action of genital mucus in its selective exclusion of abnormal and poorly motile spermatozoa thus only allowing active motile spermatozoa of normal shape into the upper genital tract. Credibility is given to the hypothesis by the variation in sperm motility during the menstrual cycle. The glycoprotein coat of spermatozoa is probably immiscible with the impenetrable form of mucus, but is able to interact favourably with ovulatory mucus. Furthermore, hydrolytic enzymes, e.g. pepsin in gastric juice, would be unable to penetrate gastric mucus and attack the underlying mucosa.

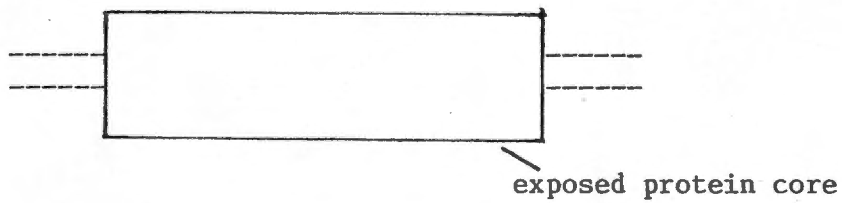
The hypothesis suggests that the intact mucus layer exploits the phenomenon of phase separation and is immiscible with solutions of most other macromolecules. This is consistent with the idea of mucus forming a protective layer and preventing the passage of enzymes, toxins and other

Figure I

The mucus glycoprotein basic unit

From mucus in Health and Disease II, 1982. Edited by Chantler R.,Elder B.J. and Elstein M. page 55.

Carbohydrate sheath



potentially harmful macromolecules to the mucosa but at the same time allowing free diffusion to small molecules.

1.4. Structure of mucus

Mucus lines all the major tracts of the body, forming a thick viscid gel layer over the epithelial surfaces. Although large differences seem to occur between the mucins of the various secretions, similarities do exist in the structure and function of mucus secretions from different sources and in different locations. Available evidence suggests the existence of a basic glycoprotein unit, characterised by what may be a common protein backbone (Silberberg et al., 1982), but differences arise in the amount and composition of the carbohydrate coat and species and organ differences may arise from this. Very large aggregates are built from the basic unit and are cross-linked by disulphide bonding, which may be either intermolecular or intramolecular. Structures having molecular weights of the order of 10-100 million have been shown to occur. The interaction of these mucins with water and with a large number of different proteins, ions, bacteria and food particles, gives rise to the special rheological properties of the mucus gel. Some generalisation is possible in discussing mucus structure and function (Silberberg et al., 1982; Meyer et al., 1977).

1.4.1. The subunit

The essential features of the glycoprotein unit which provides a common link between all mucus secretions is shown in Fig. 1. A single polypeptide chain forms a backbone of about 800 amino acids with about 63% of its length covered by carbohydrate side chains which contain 2-22 monosaccharide units (Allen, 1978; 1981). The side chains cover a single block

of the polypeptide backbone in a very dense layer, there being some 200 side chains, on the average, in this block.

1.4.2. The protein backbone

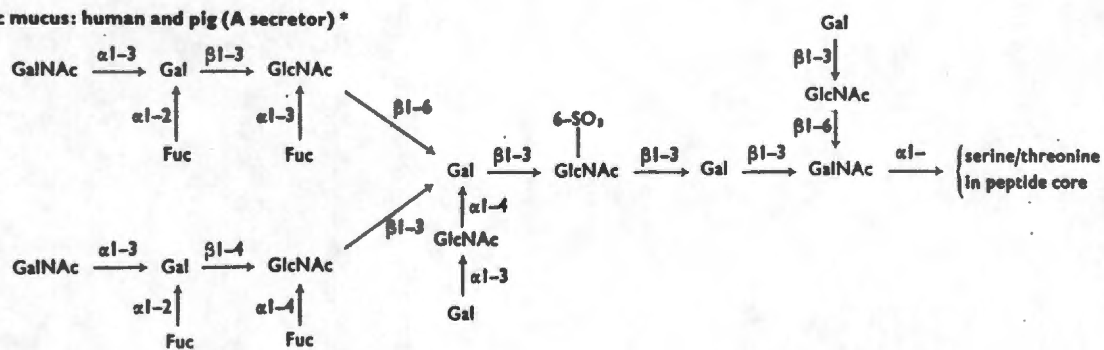
The protein backbone consists of a single polypeptide chain of amino acids. Most of this core protein is "covered" with carbohydrate side-chains but there are one or possibly two regions, consisting of 300 amino acid residues in total, that are not associated with carbohydrate. As a consequence these "naked" regions are readily susceptible to proteolytic degradation. The idea that only one 'naked' peptide region is present, as proposed in the "bottle brush" model (Allen, 1978; Scawen et al., 1977), has most support; however, there is some evidence to suggest that two "naked" regions exist, that are at each end of the polypeptide, not necessarily of the same size and this is depicted in the "rolling pin model" (Silberberg et al. 1982).(Fig.1).

The carbohydrate side-chains are linked to the backbone via O-glycosidic bonds between the monosaccharide N-acetylgalactosamine and the amino acids serine or threonine. The other amino acid that occurs predominantly in the sugar bearing polypeptide is proline. Some 16 out of 300 amino acids of the 'naked' peptide region have been shown to be cysteines. Thus the 'naked' regions are considered to be involved in the cross linking of one monomer to another. The naked region of the polypeptide is also rich in aspartic acid and other basic and acidic amino acids (Silberberg et al., 1982). Although no single, unique polypeptide chain common to all mucus glycoproteins has been found, a close resemblance between the backbone protein chains from the different mucus glycoproteins has been shown.

Figure 2.

The carbohydrate side-chain in pig and human gastric mucin.

(From Allen, 1978)

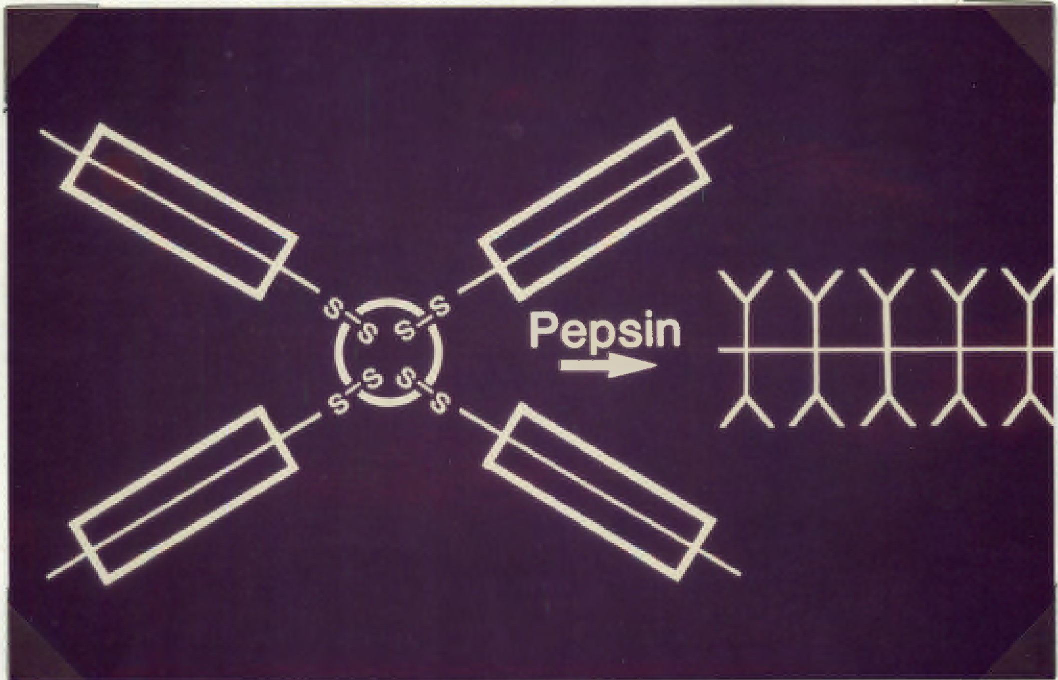


1.4.3. The carbohydrate side chains

Although, as mentioned above, there are some similarities between the polypeptide of different mucus glycoprotein, there is greater variation insofar as the carbohydrate composition is concerned (Fig. 2). The first sugar in the chain is always N-acetylgalactosamine. A feature common to many of the mucins is that they are ABH antigens for the ABO blood group system (Allen, 1978; 1981). The structure of the terminal portions of their carbohydrate side chains are the same as the blood group antigens on the surface of the red cell (Watkins, 1966). The chains are terminated either by N-acetylgalactosamine (A secretor), galactose (B secretor) or fucose linked to the 2 position of galactose (H secretor) (Allen, 1978). About 20% of people are non secretors, in which case the secretions possess neither A,B or H activity. The carbohydrate chains of the mucins end in a single galactose without the pendant fucose residue that occurs in the corresponding position in the mucins of secretors. It is not known why this profile of carbohydrate sequences is so similar to those of the glycolipids (antigenic determinants) on red blood-cell membranes. The other monosaccharides present in the side-chains include N-acetylglucosamine and sialic acid of which the exact position is unknown. In pig gastric mucin the monosaccharides constituting the side-chains have been found to be galactose:N-acetylglucosamine : fucose : N-acetylgalactosamine and sialic acid in the molar ratio of 4:3:2:1:0,2. Mucins are strongly negatively charged, especially since they contain carbohydrate-bound ester sulphate and sialic acid residues, each of which has a single negative charge (Allen, 1981). It is thought that the sialic acid residues are present at terminal positions on the carbohydrate chains, while ester sulphate

Plate 1.

Polymeric structure of pig gastric mucin
(from Physiology of GI Tract. (1981) Allen)



residues are located internally, e.g. as N-acetylglucosamine-6-sulphate in pig gastric mucin (Slomiany et al., 1972).

The hydrophilic carbohydrate side-chains ensure a strong interaction of the mucus with its aqueous environment. Since pig gastric mucins are largely carbohydrate in their composition, it is likely that the non-covalent interactions involved in gel formation take place between the polysaccharide side-chains.

1.4.4. Structure and physiology of gastrointestinal mucus

Most of the structural work on gastrointestinal mucus has been done on porcine mucus. Purified pig gastrointestinal mucus glycoprotein (mucin) is a polymeric structure of subunits joined together by disulphide bridges (Allen 1978, 1981). (Plate 1).

Either reduction of the disulphide bonds or proteolysis cleave the glycoproteins into subunits of molecular weight with consequent loss of viscosity and gel-forming properties (Allen et al., 1972; Allen, 1978; Allen et al., 1980).

Native pig gastric mucus glycoprotein (pig gastric mucin) has a molecular weight of two million and is a polymer, mode up of four glycoprotein subunits (molecular weight 500 000) joined by disulphide bridges (Allen, 1978). This single covalent entity is not dissociated in boiling 1% sodium dodecyl sulphate, 4M guanidnium chloride, 3.5M CsCl or 2M NaCl (Scawen et al., 1977, Pearson et al., 1981, Allen et al.,1982). Treatment with 0.2M mercapto-ethanol results in reduction of the disulphide bonds and the release of individual subunits. A 70 000 molecular weight polypeptide is also released on reduction of the native glycoprotein, in amounts equivalent to a molar ratio of one such protein to 4 glycoprotein subunits

(Allen et al., 1980, Pearson et al., 1981). Proteolysis also cleaves the native glycoprotein into subunits of molecular weight 5×10^5 with the loss of 40% of the total peptide but no detectable loss of carbohydrate. SDS gel electrophoresis of the papain-digested mucin shows that the 70 000 molecular weight polypeptide protein has all but disappeared after proteolytic digestion. (Pearson et al., 1981,1982)

All the above data is compatible with a model in which the four glycoprotein subunits are joined together through a polypeptide core having molecular weight 70,000 (Plate 1. pg.22). The model is supported by the detailed amino acid analysis of the components (Pearson et al., 1982) which showed that :

(1) the protein of molecular weight 70,000 and the non-glycosylated region of the mucin were both rich in cysteine residues and thus had the potential for disulphide bridge formation;

(2) reduction and then proteolysis progressively removed amino acid residues except for serine, threonine and proline. (Allen et al., 1982).

Further proteolysis or reduction of the proteolytically digested subunits did not decrease their molecular weight below 5×10^5 thus suggesting that the remaining peptide core was protected by its sheath of carbohydrate chains.

1.4.5. The structure, viscosity and gel-forming properties of mucus

The viscoelastic mucus gel has properties intermediate between those of a solid gel and a liquid (Silberberg et al., 1977). The gel matrix of pig gastric mucus is formed by the non-covalent interactions between the mucins of molecular weight 2×10^6 (Allen et al.,1977). Studies show that in

dilute solutions, the mucin is a highly expanded molecule occupying a large solution volume (Allen, 1978). In fact, an important feature of the mucus gel is its ability to retain water, thus providing a perpetual aqueous environment for the mucosal surface. Thus a complex interaction exists not only between the mucins themselves but also between the mucins and their aqueous environment. This interaction is extended to cell surfaces as well as other proteins present in the gel e.g. bacterial protein, digestive enzymes, secretory IgA, bile, pepsin and ingested food particles. The stickiness ensures that mucus adheres to the mucosal surface, providing a slimy coat to facilitate the passage of solid material through the gut. This adhesiveness enables the gel to remain firmly stuck to the mucosa and withstand considerable mechanical abuse (Allen, 1981).

Non-degradative solvents, e.g. mercapto-ethanol, dithiothreitol and sodium sulphate will dissolve the mucus gel. The viscosity for the undegraded glycoprotein in 0.2 M NaCl is 320 $ml.g^{-1}$ The viscosity of the glycoprotein solution rises from a concentration of 20 mg/ml, due to increasing non-covalent intermolecular interactions, and overlapping of their domains (Allen, 1981; Allen et al.,1983). This results in a thick, stable gel having a high molecular density, with the mucins completely filling the gel volume. An unstirred layer forms on the gastric mucosal surface which prevents HCO_3^- secreted by epithelial cells from mixing with the contents of the lumen.

The increase in viscosity can be attributed to a polyelectrolyte effect caused by the repulsion between the negatively charged groups on the glycoprotein, which causes expansion of the molecule. The repulsion, due to the sialic acid and ester sulphate residues, is only significant in

solutions of low ionic strengths, where there are insufficient cations to act as a shield between them (Allen, 1977). Within this context, the interaction of Ca^{2+} with the mucins may be important in the formation of mucus plugs in the Ca^{2+}-rich secretions of cystic fibrosis patients.

It must be remembered that the mucus gel contains more than 90% of water (H_2O). Therefore, although the mucus gel provides a considerable hindrance to the passage of larger molecules e.g. pepsin, it will be permeable to ions, e.g. H^+ and HCO_3^- . If the gastric mucin is dissociated into its subunits, the viscous and gel-forming properties of native mucus are lost (Allen et al.,1972; Allen, 1978; Pearson et al., 1980): this would explain the in vivo situation of mucus degradation through pepsin activity.

Very little is known of the direct effect of H^+ concentration on the mucus gel. It has been claimed that, at a low pH, solubilisation of the mucus gel occurs (Heatley, 1959; Schrager et al., 1978), but it is possible for this to occur as an indirect result of stimulation of pepsinolysis.

1.5 A hypothesis for protection

The Mucus-Bicarbonate Barrier

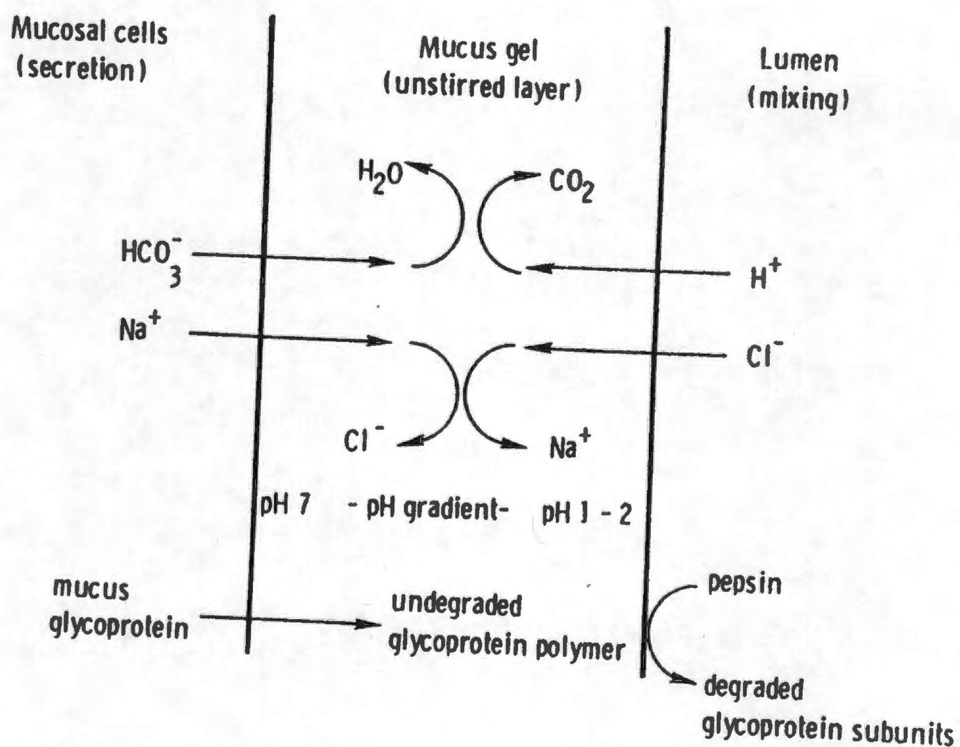
The effectiveness of mucus in vivo depends on the depth of its layer covering the mucosal surface, and on the structure of the gel. This in turn will depend on the covalent structure of the mucins, their concentration, and the strength of the non-covalent interactions between the mucin molecules (Allen et al., 1979).

The depth of the mucus gel on the mucosal surface is the result of a dynamic balance between its secretion and erosion. Degradation of the intact polymeric structure of the glycoprotein by pepsin to produce sub-

Figure 3.

A proposed model for the two component defence mechanism in the stomach

(Allen 1979)



units is a physiological phenomenon. Erosion of mucus by mechanical forces during digestion also takes place. Erosion is balanced by secretion of mucins by the mucosal cells, which have a high biosynthetic capacity and turnover rate. It seems reasonable to accept the idea that large macromolecules, eg. proteolytic enzymes would be delayed in their passage across the mucus, either because of electrostatic forces acting to impede movement through mucus (Rees et al.,1982) or contrary to Heatley's findings (1959), it has been found that the rate of transfer of hydrogen and sodium ions through mucus is delayed, being slower (3-4 times) than the rate of diffusion through unstirred water (Williams et al., 1980). Besides maintaining an unstirred layer on the epithelial surface that delays the transfer of hydrogen ions, mucus effectively immoblizes water in its interstices by the interaction of its hydrophilic carbohydrate side-chains with water molecules, thus making it unavailable for diffusion.

1.5.1. Bicarbonate ions

The gastric mucosal barrier does not provide an impenetrable barrier to acid. It is thought that its effectiveness as a protective layer is due to its ability to form an unstirred layer on the surface of the mucosa (Allen et al., 1979) and to keep restricted to the mucosal surface a layer of HCO (Flemstrom, 1981),(Fig.3). Thus a pH gradient is formed across the mucosal membrane and acid diffusing into the gel from the lumen is neutralised. This hypothesis is supported by a recent report in which by the use of microelectrodes it was shown that a pH gradient of 2.36 to 7.59 existed across the mucus or the surface of isolated rabbit gastric mucosa (Williams et al., (abstract) 1979; 1981). The barrier has also been

demonstrated in gastrectomy specimens from humans (Bahari et al., 1982).

The potential barrier role played by the extracellular layer of mucus has often attracted attention. The view that mucus acted as a physical barrier fell into disfavour when it was argued that it consists of about 95% water.

Hollander (1954),stated that mucus was one part of a two-component barrier, the other being epithelial renewal. Since the development of techniques for measuring the secretion of bicarbonate by gastric mucosa, the importance of mucus as a juxta-epithelial unstirred zone, in which luminal acid and epithelial alkali may mix, has been recognised (Rees et al., 1982). These studies have allowed the concept of a "mucus-bicarbonate" barrier to be developed (Allen et al., 1980; Rees et al., 1981).

In isolation neither the mucus layer nor the secretion of bicarbonate would be likely to afford effective protection of the gastric epithelium against damage by intraluminal contents (Rees et al., 1982). In combination, however, these secretions could produce an effective barrier with the mucus acting as an unstirred zone in which bicarbonate diffusing towards the lumen neutralises the acid diffusing towards the epithelium (Allen, 1983).

1.6. Mucus from other sources in the gastrointestinal tract of the pig.

In addition to the studies of the gastric mucins some information has been obtained about the other mucus glycoproteins found in the gastro-intestinal tract of the pig. Allen (1981),has shown that pig submaxillary mucins contain a maximum of five sugar residues per chain and about 500 such chains per molecule of molecular weight of one million.

Pig small-intestinal glycoprotein has a molecular weight of 1.7×10^6 (Mantle et al., 1981a) and is cleaved by pronase digestion into four subunits of molecular weight 4.5×10^5. Furthermore on reduction with mercapto-ethanol a protein having molecular weight 9×10^4 is released. The chemical composition of the glycoprotein is 77% carbohydrate and 21% protein, 52% of the amino acid residues being serine, threonine or proline (Mantle et al., 1981b). The molecule contains a large number of negatively charged groups such as ester sulphates and sialic acid. Although the monosaccharide content of the molecule is the same as pig gastric mucin, the molar proportions of the sugars are different, with the galactosamine content twice that of any other sugar residue.

The glycoprotein from pig colonic mucus is also an agggregate of glycoprotein subunits (Marshall et al., 1977). It has a molecular weight of 15 million and reduction of the native glycoprotein with 2-mercapto-ethanol yields a glycoprotein of molecular weight 6×10^6. The polymeric colonic mucin contains 85% carbohydrate, and the proportions of the constituent sugars are similar to those in pig gastric mucins. Substantial amounts of ester sulphate and sialic acid are present. The amino acid content is also similar to that of pig gastric mucins, and, as in most mucins secreted in the gastrointestinal tract, disulphide bridges and non-glycosylated regions of the protein core are an integral part of the quaternary structure of the native glycoprotein.

1.7. The biosynthesis, regulation and mechanism of secretion of gastro-intestinal mucus

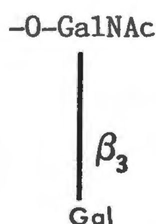
Mucin synthesis occurs on bound ribosomes associated with the endo-

plasmic reticulum and after transport to the Golgi apparatus for post-transitional processing from the epithelial cells. A radiograph following parenteral injection of radiolabeled mucosaccharide or sulphate precursors into laboratory animals have been used to study the synthetic process. These experiments have shown that the peptide backbone is assembled on membrane bound ribosomes and sugars are added subsequently as the nascent mucin core protein moves through the cell from the rough endoplasmic reticulum towards the Golgi apparatus. (Schachter et al.,1982). Glycosylation, the addition of sugars to the peptide core, takes place in the smooth endoplasmic reticulum and Golgi apparatus by the action of glycosyltransferases (Carlson, 1977). These enzymes are membrane-bound and add monosaccharide residues to the peptide backbone in sequential fashion, starting with the enzymatic attachment of an N-acetylgalactosamine residue to serine or threonine. The neutral sugars fucose and galactose are then added, followed by a terminal sialic acid. The completed mucins are then stored in membrane-bound vesicles in the supranuclear portion of the secretory cells until they are released.

In mucins the oligosacchrides are linked to the polypeptide chain only through Ser (Thr) - Gal NAc bonds. The carbohydrate side-chains of mucins show a great diversity; they may contain 1 to 22 sugars and may be linear or branched. Commonly occurring sugars are GalNAc, Gal, Fuc, GlcNAc and sialic acid. Many mucins are sulphated at a Gal or GlcNAc residue. Mucins frequently carry at the non-reducing termini of their oligosaccharides antigenic determinants for one or more of the human blood groups (Schachter et al., 1982)

Oligosaccharide synthesis is initiated by the action of UDP-GalNAc polypeptide-n-acetylgalactosaminyltransferase on the polypeptide backbone. It is presumed that the amino acid sequence of the polypeptide controls which specific serine or threonine residues are to be glycosylated and it has also been established that a certain minimum polypeptide length is essential for the transferase to operate. (Hill et al., 1977). The formation of the Ser (Thr)-GalNAc linkage does not require oligosaccharide pre-assembly but occurs by the addition of a single GalNAc residue. Once the first sugar has been incorporated into the peptide chain, the subsequent elongation is controlled by the availability of glycosyltransferases which act on the growing glycoprotein oligosaccharides.

The pre-requisite for oligosaccharide synthesis and elongation is the formation of a core. In the case of porcine gastric mucin the core is:



The key enzyme for directing the biosynthetic machinery towards oligosaccharides with this core is UDP-Gal : GalNAc-mucin β3-Galactosyltransferase (Schachter et al.,1971). The subcellular localization of this enzyme is in a Golgi subfraction. Other key transferases leading to elongated oligosaccharides with the abovementioned core appear to be N-acetylglycosaminyltransferases adding GlcNAc in β1-3, β1-4 or β1-6 linkage to the Gal residue of the Gal β1-3 GalNAc core. It is postulated that the oligosaccharide assembly requires one glycosyltransferase for every new

linkage made. It is not clear whether this glycosylation process occurs in smooth-surfaced membranes or in the Golgi apparatus.

In general, the biosynthesis of mucus glycoproteins involves the sequential transfer of individual sugars from sugar nucleotides to growing oligosaccharide side-chains of newly synthesized proteins. The reactions are catalyzed by a series of enzymes collectively called the "multi-glycosyltransferase system", or MGT system. (Carlson, 1977). Each glycosyltransferase of the MGT system requires the product of the preceding enzyme as its substrate and in turn generates the substrate for the next transferase. Thus each sugar is added separately to the growing oligosaccharide chain (Phelps, 1978).

The intracellular regulation of mucin synthesis in the intestinal tract has not been extensively studied. It is thought that regulation intracellularly is by the concentration of cyclic AMP (LaMont et al.,1977; LaMont, 1981). Also, recent morphological data suggest that calcium transport in the intestine may be associated with secretion of goblet cell mucus. Calcium has been found within goblet cells and leaves the cells with the secreted mucins. Calcium has also been shown to be firmly bound to small intestinal mucin and may contribute to the overall physical structure of mucus by binding together adjacent carboxyl groups on terminal sialic acid (Forstner et al., 1975). Another possibility is that calcium efflux is coupled to the secretion of preformed mucus granules. Cysteamine and prostaglandin $F_{2\beta}$ ($PGF_{2\beta}$) are also potent gastrin mucin secretagogues (LaMont et al., 1983).

The gastric mucosa, as well as all other intestinal epithelia, slowly releases mucus, even in the fasting or non-stimulated state (Neutra et

al., 1966). The mechanism for this basal secretion of mucus is unknown but conceivably could be secondary to tonic vagal innervation. However, the slow release of mucus type glycoproteins can be observed in organ culture of intestinal epithelium, under conditions in which vagal, hormonal, or luminal stimuli are absent (Kagnoff et al., 1972).

The major physiological regulator of gastric mucin secretion is the vagus nerve. Vagal stimulation on administration of acetylcholine or related compounds results in increased secretion from the stomach. (LaMont, 1981). The secretion of gastric mucus is not necessarily coupled with acid secretion. For example, secretin caused mucus secretion from cat stomach but had no effect on acid secretion, whereas cholinergic stimulation with carbachol caused increased release of mucus, pepsin, and acid secretion in cats (Vagne et al.,1976). Florey (1955) speculated that the superficial mucus cells of the stomach were stimulated to secrete by direct mechanical or physico-chemical factors from luminal contents, whereas secretion from deeper glands was brought about by neuronal mechanisms.

Three mechanisms of mucus release have been observed in the exposed gastric mucosa of anaesthetized, fasted dogs, by use of scanning and transmission microscopy as well as histochemical and autoradiographic techniques (Zalwesky et al.,1979). These are:

(a) Exocytosis: Release of mucus by this mechanism is slow. It is a common way for macromolecules to leave a cell, and this is the way in which mucus constantly flows on to the apical surface of the epithelial cell. The membrane of the granule joins with the plasma membrane, and the mucus flows into the gastric lumen (Moody et al., 1981). This process is

accompanied by a slight discontinuity in the plasma membrane.

(b) Apical Expulsion: By this process, mucus is released rapidly. The earliest changes observed are the gradual coalescence of granules within the cell via fusion of their membranes, and the formation of intracellular pools of mucus. Profiles of "pinched off" membrane could be observed at points of exocytosis, which opens the cellular contents to the lumen (Zalewsky et al., 1979). Mucus streams out of the cell accompanied by a few intact granules. Coalescence of granules occurs first in the centre of the cell and gradually spreads to peripheral granules.

This process is followed by a complete loss of plasma membrane, as revealed in scanning and transmission electron micrography. There is a leak of cations through these areas of gross discontinuity within the plasma membrane (Moody et al., 1981). However, the points of cell fusion are undisturbed, and the cells are still in good alignment with adjacent cells that display intact apical membranes. It is not known whether these cells can reconstitute their plasma membrane and replenish their apical mucus package.

(c) Cell exfoliation is the third mechanism by which mucus is secreted into the lumen. Here the entire cell is extruded into the lumen and may provide, in addition to apical explusion, a second mechanism to rid the mucosa of senescent epithelial cells. Apical expulsion and cell exfoliation involve the most aged cells at the crests of the foveolae where neutral glycoproteins predominate. Exocytosis is the only mechanism observed in the mucosal pits where sulphated glycoproteins are concentrated (Zalewsky et al., 1979).

Several other features of the surface cell deserve special emphasis. The apical cluster of mucus granules is surrounded by a circumferential accumulation of mitochondria. This implies a need for energy in the mucus secretory process (Moody et al., 1981). It is possible that the mitochondria support the synthesis as well as the release of mucus. Their lateral position also suggests a role in providing energy for ion transport into and out of the lateral intercellular spaces. They may also serve as a reservoir for calcium ions, thought to be important in the release of membrane-bound intracellular substances. In this scheme, the mitochondria would provide energy for the apical movement of granules and the ultimate evacuation of their contents into the gastric lumen.

1.8. Histology of the pig stomach

1.8.1. Introduction.

The stomach is a dilated,specialised segment of the digestive tract between the oesophagus and small intestine that serves to store as well as to process food for absorption by the small intestine (Ito, 1981). It is specialised for the enzymatic and hydrolytic breakdown of food into digestible nutrients.

Although the stomachs of vertebrates show common structural features, their gross morphology varies. The varying morphology is related to species differences in body size and shape, need for food storage, nature of diet and frequency of food intake.

1.8.2. The Pig as an Experimental Model in Gastric Ulceration.

Despite recent advances in treatment, peptic ulceration in humans still constitutes a considerable clinical problem. The domestic pig *Sus*

scrofa domestica, is well suited as an experimental model for the study of peptic ulceration, since pigs have been shown to develop gastric ulceration spontaneously (Watson et al., 1978) in the pars oesophagea with a reported natural incidence of 25% or higher (Terblanche et al.,1978).

Bile duct ligation has become an established technique to induce ulceration of the stratified squamous epithelium of the pars oesophagea of the the pig's stomach (Arnot, 1974). In my specific study of the physical and chemical changes that occur in mucus associated with ulcerated states the pig was used as a model because of the close similarities between pig and human gastric glycoprotein (Allen,1981). Also since large amounts of secreted mucus on the surface of the mucosa were readily obtainable for the purpose of extraction and purification prior to biochemical analysis, the pig stomach was suitable for such a study. The availability of human mucus is severely limited, for obvious reasons.

The stomach of the pig is large, that of a 40kg specimen having a capacity of up to 5 litres when full. It lies almost transversely in the upper abdomen (Arnot,1974). The lesser curvature is short, measuring 10-15 cm while the greater curvature is considerably longer. Compared with the rest of the gastrointestinal tract, its muscle wall is thick, especially in the antral region. The muscle wall of the cardiac gland region is thinner, and that of the cardiac diverticulum measures only 2 mm in thickness.

Despite widespread reports regarding the histological appearance of the pig stomach, little information is available on the microstructure of the stomach of healthy pigs. What little is known is based on work done during the previous century (Lombard, unpublished) and early this century (Sloss, 1932) and again a little later (Sloss, 1952).

The Division of Histology in the Department of Anatomy at the University of Cape Town has embarked on a comprehensive investigation of the microsctructure of the stomach of various members of the porcine family. Preliminary observations obtained by light microscopy will be reported here. Histological observations on the distribution of mucus in the different regions of the normal pig stomach will also be reported.

Pigs aged between 6-8 weeks and weighing 16-20 kg, of either sex and mixed genetic origin, were reared on conventional pig growth rations and were healthy at the time of sacrifice.

Two distinct areas of the stomach were distinguishable macroscopically:

(a) A non-glandular area lying around the oesophageal gastric orifice.

(b) A glandular region within which were two distinct additional morphological features, the cardiac diverticulum and the torus pyloricus. The cardiac diverticulum, is a pouch-like dilatation occupying the proximal part of the greater curvature. The torus pyloricus is a small oval, pedunculated structure attached to the wall of the lesser curvature and protruding into the lumen of the pylorus. Functionally, the torus in conjunction with the pyloric sphincter, acts as a plug to close the lumen of the pylorus.

Externally, the stomach can be divided into three sections (Plates 2 and 3). Adjacent to the oesophageal gastric junction, lies the bulbous, thin-walled cardiac gland area (Lombard, personal communication), from which a pouch-like dilatation the "cardiac diverticulum", projects dorsally. It is thought to be a storage place for less digestible substances (Arnot, 1974), since it has been found to contain fragments of

straw, hair, pieces of string and plastic material.

The body of the stomach tapers downwards to join the antrum which narrows to the pylorus.

1.8.3. The different anatotomical areas of the pig stomach

(See Plates 2 & 3)

Glandular area

The surface epithelium of the glandular regions is morphologically uniform throughout. The mucus membrane of each region is similar in micro-structure to that of the human stomach, although differing individually in relative depths of their pit and glands. Endocrine cells (Fujita et al., 1981), occur in all regions of the glandular mucosa. On the mucosal surface the cells are tall columnar (with a height of 20-40 μm and a width of 6 μm) whilst in the pits and isthmus they are lower and broader (Helander,1981).

Cardia

Patches of lymphoid tissue are the main diagnostic feature of the cardia of the pig stomach. Macroscopically, the cardia appeared pitted though not as obviously irregular as the body region (Warton, personal communication, 1983). The mucous membrane of the cardia is much thinner than in the body region.

The cardiac glands are lined by cells which are serous rather than mucous in appearance and are referred to as cardiac serous cells. These serous cells have round nuclei which are vesicular and more or less centrally located (normally mucus cells have a pale cytoplasm with flattened disc shaped nuclei situated at the base of the cell).

Plate 2

The pig stomach (normal) opened along the greater curvature

A. The pyloric antrum

B. The body area

C. The cardiac gland area

P. The pars oesophagea

(By courtesy of Dr. Chris Warton)

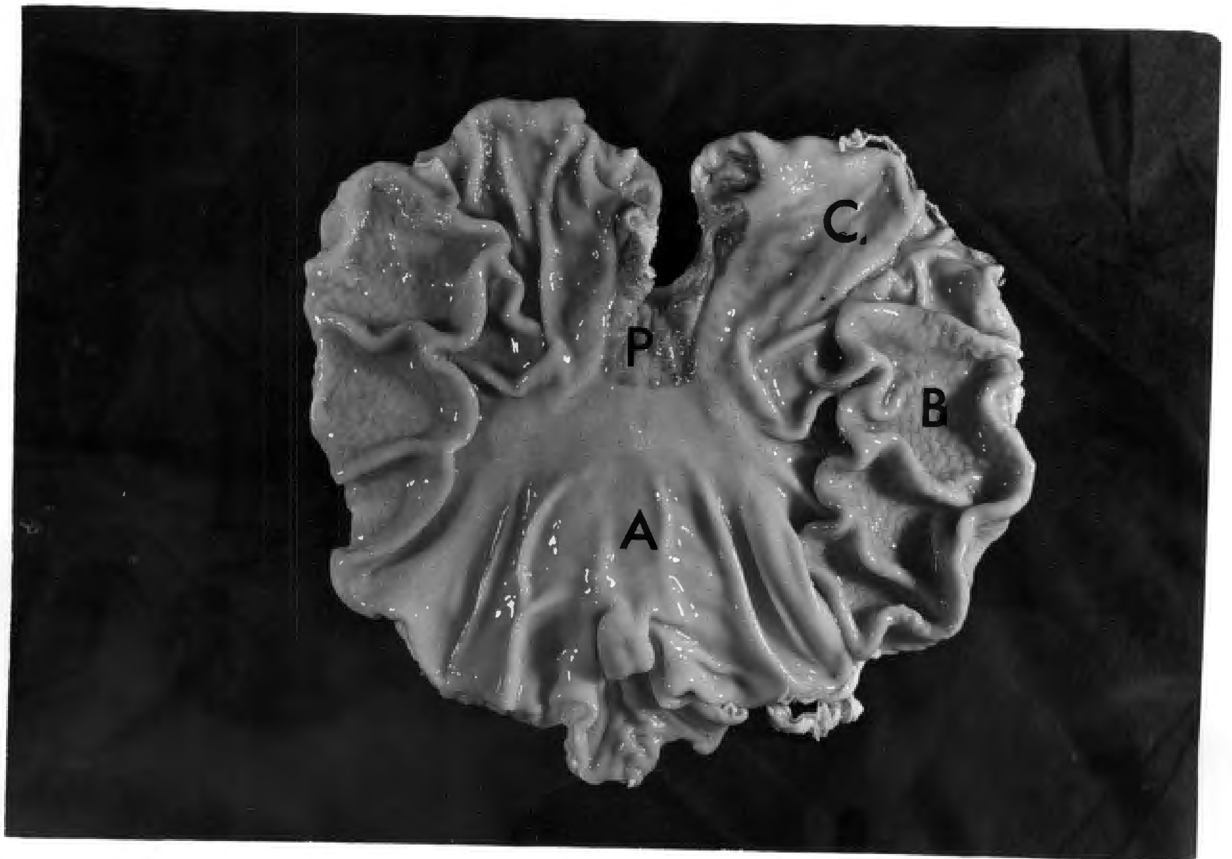


Plate 3

The pig stomach (ulcerated) opened along the greater curvature.

Arrow points to ulcerated region.

Interspersed among the cardiac serous cells are endocrine cells, more than 50% of which are X-cells in the porcine cardiac gland area (Solcia, 1981).

Body

On a macroscopic level the body is dimpled in appearance and much thicker than the cardiac gland area. The rugae or folds in the body region are taller than those in the cardia. In both the cardiac gland region and the pylorus the rugae run longitudinally and appear in a ring-like formation.

The surface mucus cells extend a short distance into the glands. Mucus neck cells, parietal and chief cells constitute the remainder of the glands. In the proximal body region the gastric glands have been shown to have many parietal and fewer chief cells; however, in the distal half of the body the glands have more chief cells (Warton, personal communication). Numerous endocrine cells are interspersed amongst the other cells (Ito, 1981).

Pyloric gland area (Antrum)

This area is actually the region covering the entire lesser curvature and distal part of the greater curvature. Macroscopically, it is smooth, flat and whitish in appearance. It has longitudinal rugae, and its mucous membrane is thinner than that of the body.

The glands of the pyloric mucosa have differing numbers of morphologically "mucous" or "serous" cells in different areas. In the proximal antral region the "mucous" cells appear typically at the base of the glands. The remainder of the glands is made up of "serous" cells morpho-

logically indistinguishable from cardiac serous cells. In the glands of the middle region the mucous cells extend further up from the base of the glands. In the distal region all the cells are mucous in type and the glands become longer and the epithelium thickens.

The torus pyloricus is a small oval mass projecting into the lumen of the pyloric canal, covered with typical pyloric mucosa and underneath this is a mass of smooth muscle and adipose tissue.

1.8.4. Mucus production in different areas of the porcine stomach

Different regions of the pig stomach viz. the cardia, body, middle pyloric region and distal pyloric region may be identified on the basis of the distribution of mucins (Lombard, 1982, personal communication). For convenience, general observation of the type of mucus is made at 3 levels, i.e.

(i) The luminal surface including pits.

(ii) The upper and middle 2/3 of the glands.

(iii) The base of the glands.

All mucus cells stained to some degree with PAS indicating the presence of neutral glycoproteins. Acidic mucins stained with Alcian Blue.

(i) Luminal Surface of Pits.

With the exception of the cardia and adjacent portion of the body regions the surface mucus cells of all regions contained neutral mucins. The cells on the luminal surface of the cardia contained predominantly non-sulphated acidic mucins while the cells of the pits and the proximal portion of the glands contained sulphated mucins. This distribution extended into the adjoining region. However, the surface cells gave a

heterogenous picture, giving way to predominantly neutral mucins in the area adjoining the pyloric antrum.

(ii) <u>The upper and middle two-thirds of the glands.</u>

In the pyloric antrum, the mucins were found to be mixed, showing the presence of neutral, acidic sulphated and non-sulphated mucins. In the cardia and body, the mucus cells in the most proximal portion of the glands contained sulphated acidic mucins, giving way to neutral mucins below.

(iii) <u>The Basal portion of the Glands.</u>

With the exception of the pyloric antrum proper the loss of the glands in all regions contained neutral mucins. On this basis the pyloric antrum can be divided into three distinct forms.

(a) The proximal pyloric region with neutral mucins.

(b) The mid pyloric region with predominantly sulphated mucin.

(c) The distal pyloric region with neutral mucins.

The basal portion of the glands in this region were continuous with the Brunner's glands of the duodenum through the muscularis mucosae.

1.9. Definition of an ulcer

1.9.1. What is gastric ulceration?

Introduction

This thesis would be incomplete without a definition of the term "ulcer". The term is used widely and it has become difficult to evaluate experimental observations, case reports and autopsy protocols because of the indiscriminate use of the term (Ivy et al.,1950).

The authors cite the example of Sterlin, who in 1920 observed duodenal ulcers in a dog with a bilateral vagotomy and a removal of the nerves of the coeliac plexus. On microscopic examination, it was revealed that what was thought to be an ulcer was actually a traumatic mechanical injury of the mucosa brought on during the postmortem. Sterlin emphasized the need for microscopic studies in future evaluations.

Normal structures have been mistaken many times for chronic ulcers. In 1938, it was reported that circumscribed depressions of the duodenal mucosa in dogs due to the location of lymph follicles and Peyer's patches were mistaken for ulcers by inexperienced observers. The colloquial expression "freshman ulcers" was coined as a result.

"Ulcer" is a term applicable not only to the gastrointestinal tract but also to the skin, cornea and mucous membranes. At a superficial glance, there is a resemblance between ordinary erosions (abrasions) and ulcers. Thus it has become important to define ulcers more specifically to bring out the important differences between the various closely similar abnormalities.

1.9.2.A General Definition of an Ulcer

Amongst the very large number of definitions in the literature, Ivy et al.,(1950) found that of Karsner (1942) most appropriate. An "ulcer may be defined as an interruption of surface continuity with an inflammatory base"..... "Ulcers may be found on any surface of the body and may be due to traumatic destruction of the surface, or result from degeneration, necrosis or inflammation in the neighbourhood, which leads to the sloughing of the injured superficial tissue from the underlying parts."...."In more prolonged types of ulcers, the base is likely to show, in addition to nervous exudation, considerable infiltration by the cells seen late in the course of inflammation."

This is clearly different from an abrasion which is superficial and confined to the epithelium, resulting from mild trauma, such as falling and scraping the skin. An ulcer is characterised by a loss of continuity of the skin or mucous membrane, has depth and extends into the deeper structures of the skin or mucous membrane. It shows a circumscribed complete loss of epithelial elements, is associated with inflammation and heals with the formation of scar tissue.

1.9.3.Definition of an Ulcer as Applied to the Stomach

The next step is the application of the above definition to the specific organ of our concern, namely, the stomach. The wall of the stomach is composed of several layers. The mucosa of the stomach consists of two layers, the surface epithelium and the glandular layer. The glandular layer contains the gastric glands which are surrounded by connective tissue, blood vessels, lymphatic vessels and nerves. The

muscularis mucosa lies external to the layer of glands.

An ulcer may be diagnosed only if the continuity of the mucosa is lost completely and the defect extends through the muscularis mucosa and frequently through the remaining layers. If the defect does not extend to the muscularis mucosa, it is not an ulcer but an erosion. Thus, the major distinction between an ulcer and an erosion is depth. The erosion heals by epithelial regeneration without scar formation whilst the ulcer heals by formation of the granulation tissue. By international convention, a gastric ulcer is a breach in the gastric mucosa extending through the muscularis mucosa (Segal, 1983).

1.9.4. Usage of the Term "Peptic Ulcer"

The expression "peptic ulcer" has been used widely for several decades to refer to benign, non-specific ulcers of the lower portion of the oesophagus, stomach, duodenum and jejunum.

The usage of the term has an interesting historical background (Ivy, et al.,1950). The acidity of gastric contents was demonstrated in 1692. In 1824, Prout discovered that the acidity of the gastric juice was due to hydrochloric acid but this was only accepted in 1856. Pepsin in gastric juice was discovered in 1836.

In 1852-1853, workers proposed that gastric ulcers were due to the corrosive action of gastric juice acting on an area of mucosa rendered susceptible by venous stasis caused by local spasm of the gastric musculature or to thrombosis of the blood supply of the ulcerated area.

The expression "peptic ulcer" was first used in 1882 by Henirich Quinke. It was widely adopted because it referred appropriately to non specific

ulcers found in those regions exposed to gastric juice. It also implied that the ulcer was caused by the peptic activity of gastric juice. Prior to the use of the expression "peptic", ulcers were described as "round", "simple", "corrosive" and "perforating". Although "peptic" was more descriptive, its use has declined over the years after the recommendation of Shay (Ivy et al., 1950) that ulcers should be designated according to their anatomic location. Gastric and duodenal ulcers differ and are preferably regarded as distinct entities, rather than being categorically defined as "peptic", a more ambiguous expression. Gastric and duodenal ulcers differ in their pathology and etiology. It would be important to regard them as distinct entities. Placing them into the category of "peptic ulcers" would fail to highlight the major differences between them.

1.10. Mucus in disease

The study of glycoproteins has intensified with the discovery of their important functions in relation to disease. For example, most normal cells in culture stop growing when they touch each other (contact inhibition), but cancer cells grow without restraint, and this seems to relate to the properties of their cell surface glycoproteins. Exciting new developments are taking place in the relationship between the glycoproteins of mucus and those of the cell surface and the possible relationship of the latter to carcinogenesis (Elstein et al., 1977).

The field of glycoconjugate research has become very active recently as evidence accumulates implicating these macromolecules in many disease conditions. Many tissue culture studies have underlined the importance of cell-surface glycoproteins and/or proteoglycans in regard to cessation of

growth of normal cells once they become confluent. This latter property of normal cells is in stark contrast to what is seen when cells are transformed by chemical or viral means, resulting in changes to the cell surface glycoproteins (Hawtrey et al., 1974).

It has been suggested that the rate and form of mucus secretion are a reflection of cell turnover and cell life. The mature cell produces abundant mucus with its protective, lubricant and transportation roles in the tissues. On the other hand rapidly growing cells in hyperplastic conditions produce less mucus of a different type, possibly related to cell transformation and the development of malignancy. The degradation of mucus may be a central event in the pathology of the gastric mucosa (Schrager et al., 1978). Insight into the mechanism of such changes may be of relevance to the understanding of the development of certain pathological conditions.

Increased acidity and pepsin alter the organisation of mucus (Schrager et al., 1978), resulting in an increase in glycoprotein species of lower molecular weight relative to native large glycoprotein on the mucosal surface (Younan et al.,1982). The presence of glycoprotein of lower molecular weight, a feature of both human and pig gastric mucus, is thought to be the result of pepsin action _in vivo_ (Pearson et al.,1980). _In vitro_ studies using papain, pepsin, pronase and trypsin have shown a decrease in the viscosity of gastric mucus following digestion (Allen et al., 1974). Reductive cleavage with 2-mercapto-ethanol caused a large decrease in viscosity of water soluble mucus. Both enzymatic digestion and reductive cleavage of the parent molecule resulted in the formation of four subunits of equal size (Scawen et al., 1977) with concomitant loss of viscosity and gel-forming properties of the native molecule.

It seems likely that the mucus gel on the surface of the mucosa may contain both native and degraded glycoprotein (Pearson et al., 1980), with the degraded product then entering the lumen. The unstirred layer of mucus on the surface of the mucosa is of constant thickness due to a dynamic balance between secretion and breakdown (Allen et al., 1980). An increased content of degraded glycoprotein subunits on the surface of the human gastric mucosa, was accompanied by decreased secretion and increased pepsin degradation in peptic gastric ulceration. Features of this condition are high acid and pepsin levels and as mentioned, this is associated with an increased presence of degraded glycoprotein subunits on the surface of human gastric mucosa. This increase in degraded subunits may result from either decreased secretion of new "replacement" intact mucin or increased degradation of the whole molecule. (Schrager et al., 1978; Yeoman et al., 1982). Furthermore the increase in degraded mucin was accompanied by decreased viscosity of the mucin.

The significance of these findings is that in chronic peptic ulceration there is a decrease in the capacity of the mucosa to form polymeric glycoprotein, since intactness of the native molecule is essential for gel formation it is possible that under pathological conditions, such as ulceration or carcinoma, the destruction of polymer integrity would have a marked effect on the gelation of mucus (Pearson et al., 1980).

There is little doubt of the importance of altered mucins in the aetiology of gastrointestinal disease, particularly ulceration. All the evidence to date has indicated a breakdown of the mucus gel layer when the erosion of the mucosal surface has occurred. Whether the erosion of the

mucosa and the occurrence of ulceration was due to an intrinsic weakness of the mucus gel, or the changes of mucus observed were a consequence of gastric erosion has not been established.

It has become essential to investigate altered patterns of both the carbohydrate side-chains and the amino acid composition of the mucin in diseased states. Only then can the role of mucus in the etiology of gastric ulceration be more clearly defined. Comparisons have been made of the ratios between the various monosaccharides associated with glycoprotein in gastric washouts from ulcer patients and control subjects (Glass et al., 1977; Schrager et al., 1978). It has been found that there is a decrease in glycoprotein-bound sialic acid in the gastric fluid from ulcer patients as compared to controls (Domschke et al., 1972). In work of our own, which will be discussed later in detail, we have found a marked reduction in fucose content of mucin obtained from animals with positive ulceration as compared to control animals (See Chapter 4).

Blood groups such as ABH have been shown to exert some influence on peoples' susceptibility to peptic ulcer, gastric cancer and one of it precursors, pernicious anaemia (Glass et al., 1978; Schrager et al., 1978). People with blood group O were especially liable to peptic ulcer (Allen, 1978) and also showed an increased frequency of duodenal ulceration (Glass et al., 1962). However, the latter was also apparent for non-secretors of ABH groups. In our studies, the pigs used were entirely of the O blood group, which was confirmed by the fact that neither N-acetylgalactosamine (A blood group) nor galactose (B blood group) was present as a terminal sugar (Allen, 1978;1981). Therefore a possibility exists that the genes controlling blood groups are linked to those predisposing to peptic

ulceration (Schrager et al., 1978).

There is histochemical and biochemical evidence for changes in the mucus glycoproteins associated with malignant mucosa or intestinalised gastric mucosa (Glass et al., 1977; Schrager et al., 1978; Allen, 1981), since antigenic differences between sulphated glycoproteins from normal and carcinomatous gastric secretions have been reported (Hakkinen et al.,1968).

Neoplastic glycoproteins differ from the glycoproteins isolated from normal gastric mucosa only in the quantitative relationship of various carbohydrate components (Glass et al., 1977). It has been reported that less cytoplasmic mucus was found in gastric cancer cells than in normal gastric surface epithelium or in goblet cells of the intestinalised mucosa. There was a distinct reduction in the proportion of the carbohydrate fraction of the mucin from human colonic neoplasm as compared with that from adjacent normal colonic mucosa (Kim et al.,1974). The contents of sialic acid, fucose, N-acetylglucosamine and N-acetylgalactosamine were lower and furthermore there were reduced activities of certain specific glycosyltransferases (Boland et al.,1982).

The difference between the mucin of colonic cancer from that in the normal colon has also been detected by lectin binding characteristics (Boland et al., 1982). Fluorescein isothiocyanate (FITC) conjugates of *Dolichos biflorus* agglutinin (DBA) were found to bind mucin from normal colon and peanut agglutinin (PNA) to bind mucin of colonic cancer. A converse relationship existed between the amount of binding to either lectin in the normal state and in colonic cancer.

A sulphated glycoprotein antigen with a high ratio of N-acetylgalactosamine to other sugars has been isolated from gastric tumours. This is also true for glycoprotein from intestinal metaplasia. These findings suggest the occurrence of short carbohydrate side chains since this sugar is found only at terminal positions in the side-chains.

A comparative study between the glycoproteins isolated from normal and malignant areas of the same surgical specimens revealed some interesting differences. Amino acid analysis yielded a lower content of serine compared to threonine (Schrager et al., 1978) in the samples from neoplastic mucosa, and there was also an increase in the ester sulphate and sialic acid content in such samples when compared with those isolated from normal gastric mucosa (Schrager et al., 1973). The ratios of the major monosaccharides differed significantly from those of the normal glycoproteins. Also, there was a deviation of the blood group specificity of "malignant" glycoproteins from the host red cells involving A,B, and O blood groups. This apparent change in the secretor status of patients with gastric carcinoma is further evidence for changes in the structure of the sugar chains of their mucus glycoproteins, e.g. in some cases where the host's red cells were O, the gastric mucus indicated an A secretor (Allen, 1981). Histological studies show that a switch from sulphated mucins to sialomucins may be associated with colonic malignancy (Forstner, 1978). In a study of rat colonic carcinoma induced by chemical carcinogens it was observed that malignant transformation was accompanied by an increase in secreted sialomucins relative to sulphomucins. This suggests that intestinal malignancy may be associated with defects in mucin biosynthesis,

particularly in the terminal steps of glycosylation and sulphation. Malignant tissue from the rectum has been shown to contain very high properties of acidic, non-sulphated mucosubstances (Subbuswamy, 1971). The author has suggested that sulphated mucins in these areas possibly controlled cell division, and that reduced secretion tipped the balance towards malignancy. If nothing else, this switch from sulphated mucins to sialomucins may become diagnostically significant and important (Glass, 1977).

Villous adenomas in colon have been reported to secrete large quantities of mucus (375-2000 ml/day). The most obvious difference between normal and malignant mucins of the colon is the increase of N-acetyl-glucosamine to N-acetylgalactosamine from a small amount in the normal to 3 times as much in the malignancy (Schrager et al. 1978). Although the mucins from both the normal and the malignant states contain the full complement of amino acids and monosaccharides, the "malignant" mucins have a much wider variation of monosaccharide content and different ratios of threonine to serine and of leucine to isoleucine than have the normal mucins (Schrager et al., 1978). Other workers have reported a change in the immunological properties of the mucin from cancerous colons, with a 40% (by weight) increase in aspartic acid and a decrease in threonine content, together with a lower density of cabohydrate chains per molecule (Allen, 1981).

The importance of the above findings is revealed by the possibility of a new classification of tumours based on the composition of mucins secreted by malignant cells: their monosaccharide ratios, amino acid composition,

(the threonine : serine ratio), sialic acid content and antigenic properties are all possible distinguishing criteria.

To account for the frequent association of venous thrombosis and mucus-producing carcinomas of human lung, stomach, and colon, a pathological role in promoting blood coagulation has been suggested for mucins (Forstner, 1978). It was found that extracts of intestinal mucus secretions would activate factor X of the clotting cascade _in vitro_. If injected into rabbits, the extract caused intravascular coagulation and thrombosis. They have speculated that in malignancy, epithelial permeability to mucins or their metabolites is increased, and absorption of mucins into the blood stream leads to hypercoaguality and venous thrombosis.

The changes in glycoprotein secretion under pathological conditons correlate with the proportions of the various types of glycoprotein found. Experimental studies of the airway surface showed an increase in the number of secretory cells caused by irritation, in the absence of infection (Jones et al., 1978). Secretory cell hyperplasia is a feature of tobacco smoke inhalation as early as 20 hours after exposure. After 3 days of exposure, the increase of secretory cells in the lower trachea and bronchi was found to be significant. These cells contain mainly acid glycoprotein.

Cystic Fibrosis

In cystic fibrosis mucus producing cells all over the body show hyperplasia and hypertrophy. The secreted mucus in patients with cystic fibrosis has an increased viscosity and forms insoluble plugs (Forstner, 1978). The mucus secretions are associated with electrolyte disorders in

the gastrointestinal and respiratory tracts (Glass, 1977). An investigation of the intestinal and other epithelial mucins in cystic fibrosis shows an increase in cytoplasmic and extracellular mucus. Tissue culture studies using double isotope experiments with both ^{3}[H]-glucosamine and [^{35}S]-sulphfate as precursors of mucus glycoprotein with material from normal subjects and patients with cystic fibrosis, showed a shift to more acidic components in the latter's purified mucin with a higher level of sulphation (Frates et al.,1983). The degree of sulphation of the glycoprotein in the mucus secreting cells of the airway surface is increased both in chronic bronchitis and in cystic fibrosis (Jones et al. 1978). In the same conditions, once lung infection has occurred, there is an increase in the proportion of mucus cells of the gland containing neuraminidase-resistant sialyated glycoprotein. Cystic fibrosis intestinal mucin has been found to be dense and more highly glycosylated than normal mucin and there are also increases of sulphate content and oligosaccharide chain lengths. (Wesley et al., 1983). Very recently, it was reported that the biochemical defect in the gastric mucus of cystic fibrosis patients was the presence of covalently bound fatty acids to mucus glycoprotein (Slomiany et al., 1983). This applied also to salivary and tracheobronchial secretions. It was proposed that the covalently attached fatty acids contributed to the proteolytic resistance of gastric mucus glycoprotein in this disease.

Cholesterol mucins

Mucin has been identified in the matrix of cholesterol gallstones (Smith et al., 1983). It is the main constituent of the mucus gel lining

the epithelial surface of the gall-bladder and provides a diffusion barrier against the potentially toxic compounds in the bile. However, experimental work stongly suggests that gall-bladder mucin and bilirubin form complexes and function as nucleating agents for cholesterol gallstones (Lamont, 1981).

Ulcerative colitis and Crohn's disease

A decrease in sulphated and non sulphated acidic mucins has been reported in ulcerative colitis (Glass, 1977). There is an overall decrease in the amount of mucins secreted in the majority of cases of ulcerative colitis, as opposed to Crohn's disease, a finding of important diagnostic value. The carbohydrate composition and the molar ratios of the constituent sugars do not change in colitis but the lengths of the carbohydrate chains are reduced to half of those in normal mucins. The number of serine and threonine residues are also reduced by half and this decreases the number of sites available for the formation of O-glycosidic linkages. Abnormalities in composition of the carbohydrate components, such as the finding of high mannose levels, have been shown in colitis (Ehsanullah et al., 1982). Whether this occurs as a result of a compositional change of colonic mucosal glycoproteins or is due to a leak of serum alpha-globulins is unknown.

Glucosamine synthetase activity was found to be increased by about 50% in intestinal biopsies of patients with Crohn's disease (Glass et al., 1977).

1.11. Drug effects on mucus and its secretion

The maintenance of a healthy gastric mucosal lining in normal physio-

logical terms depends on a balance between the forces of aggression, namely hydrochloric acid and pepsin, and the state of the barrier to these factors, of which mucus is now being recognised as an essential component. Abnormalities in the secretion of mucus are associated with a number of disease states (Parke, 1978). The clinical importance of mucus becomes obvious when the pharmacological effects of a variety of drugs, chemicals and hormones on its synthesis, secretion and consistency are noted.

Aspirin.

The effects of aspirin on the mucus and mucosa have been well documented. It was found that when aspirin was administered to dogs with vagally denervated gastric antral pouches, there was both a substantial decrease in the amount of mucus secreted and a reduction in the number of oligosaccharide side-chains of the mucin, with a specific decrease in the acetylated sugars and sialic acid. (Menguy, 1965, 1969). In humans, aspirin also reduced the amount of mucus adherent to the mucosa of the stomach, either by shedding into the lumen or by decreased secretion (Parke,1978). Aspirin has been reported to inhibit mucus biosynthesis at low concentrations both in man and in experimental animals (Allen et al., 1980).

Aspirin is regarded as a major causative agent of gastric and duodenal ulcers (Parke, 1978), as shown by clinical studies of the mechanism of gastrointestinal inflammation. The main features of this condition were the impairment of mucus synthesis and loss of integrity of the gastric bicarbonate barrier. The oral administration of aspirin in rats resulted in a significant reduction in the rate of gastric mucus synthesis, as

measured by the incorporation of N-acetyl-^{3}[H]-glucosamine and the development of gastric erosions. The inhibition of incorporation of ^{3}[H]-threonine into mucin synthesized by the human stomach, and the reduction in the rates of incorporation of [^{14}C]-glucosamine and [^{14}C]-leucine into rat small intestine were other reported effects of aspirin (Parke, 1978). Its topical application has also caused the shedding of the adherent mucus followed by the inhibition of cellular respiration. The inhibition in the biosynthesis of mucins, proteins and nucleic acids was coupled with decreased prostaglandin synthetase activity, which resulted in a lowered tissue prostaglandin activity, leading to decreased HCO_3^- secretion (Allen et al., 1980; Rees et al.,1980; Johansson et al., 1982,). Aspirin has been shown to uncouple oxidative phosphorylation and thus reduce the overall ATP content of gastric mucosa. (Guth,1982).

Anti inflammatories and Steroids.

Anti-inflammatory drugs have been shown to cause gastrointestinal inflammation, associated with impaired mucus production, erosions of the mucosa and haemorrhage (Parke, 1978).

The drugs that have been implicated, in order of decreasing toxicity, are: indomethacin, diclofenac, phenylbutazone, aspirin, oxyphenbutazone and salicylate. All these drugs inhibit the synthesis of mucin by impairment of glycosylation (Parke et al., 1977). The anti-rheumatic drugs phenyl-butazone and indomethacin, which when administered to dogs, resulted in the synthesis and secretion of a mucin molecule which was less resistant to pepsin and trypsin (Menguy, 1969). Indomethacin and fenclofenac are potent inhibitors of HCO_3^- secretion in the fundus and antrum of man (Garner et

al., 1979). The relationship of adrenocortical steroids to acute gastric mucosal injury, although well documented, remains controversial (Domschke et al., 1984). Like aspirin, the ability of adrenocortical steroids to injure the gastric mucosa has not been satisfactorily explained by the acid peptic factor. It was postulated that these factors induce gastric ulceration, at least, in part, from a decreased mucus secretion (Fenster, 1973).

Carcinogenic Agents.

Malignant tumours are characterised either by a large decrease or an increase in mucin synthesis and secretion (Parke et al, 1977). Biopsy material taken from patients with gastric carcinoma had very low activities of the mucosal glycosyl transferases as measured by the incorporation of N-acetyl- [H]- glucosamine. This incorporation was abnormally low in most areas of the stomach, especially in the vicinity of the carcinoma.

In vitro studies of malignant cells transformed by carcinogenic chemicals show that malignancy is the result of at least 4 processes, namely:

1) metabolic activation of the chemical carcinogen.
2) damage of the endoplasmic reticulum by the proximate carcinogen leading to degranulation.
3) switchover of cellular metabolism to cell growth at the expense of glycoprotein synthesis.
4) alkylation of the DNA and erroneous transcription.

In the light of the above information, it is interesting to note that drugs like carbenoxolone stabilise the rough endoplasmic reticulum, and may eventually have a role in the treatment of epithelial cancer (Parke, 1978).

Carbenoxolone

Carbenoxolone, a triterpenoid derivative, marketed as Biogastrone, is a drug which accelerates the healing of gastric ulcer (Shillingford et al., 1974). Conflicting evidence has been put forward by different workers regarding the pharmacological action of carbenoxolone. Shillingford et al.,(1974) reported a marked increase in mucus secretion in human stomach, surgically removed after treatment with carbenoxolone. They also observed an increase in N-acetyl- ^{3}[H]-glucosamine incorporation and a decrease in [^{14}C]-galactose incorporation in rats dosed orally for 7 days with carbenoxolone. On the other hand van Huis et al., (1981) found no change in the sugar composition of mucins of carbenoxolone treated rats. They reported an increase in the ^{3}[H] thymidine labelled nuclei per fundic pit, implying a higher number of mitotically active cells. This rise in the number of mucus- secreting cells results in increase in the total amount of mucin per stomach. Whilst early work suggested that carbenoxolone reduced the extent of mucosal damage produced by aspirin in dogs (Parke, 1976), more recently other workers showed that although carbenoxolone did indeed accelerate the healing of gastric ulcers and prevented erosions caused by stress, it did not prevent erosions due to aspirin (Van Huis et al., 1981).

More effects reported for carbenoxolone include decreased back-diffusion of hydrogen ions from the gastric pouch of dogs (Parke,1976) and an effect on prostaglandin metabolism resulting in an enhancement of the micro-circulation of the gastrointestinal epithelium, thus increasing the supply of nutrients for mucus synthesis. Carbenoxolone has also been postulated to inhibit the intraluminal action of pepsins (Roberts et al.,

1973). Finally some suggested mechanisms for the effect of carbenoxolone are an increase of microsomial glycosyl transferase, inhibition of 3 : 5 cylic AMP phosphodiesterase activity in the gastric mucosa and inhibition of gastric prostaglandin dehydrogenase and 1,3Δ reductase, enzymes known to deactivate prostaglandins (Guslandi, 1980).

Cimetidine

The medical treatment of peptic ulcer is mostly directed to the neutralisation or inhibition of peptic acid secretion. The discovery of H_2 -receptor antagonists has resulted in an increase in the use of agents that inhibit hydrochloric acid secretion. Cimetidine, undoubtedly the most widely used H^2-receptor antagonist, has been shown, however, to be capable of causing damage to gastric mucus (Guslandi, 1978). Thus it has been proposed that the recurrence of peptic ulcer in cimetidine healed patients when even after a long-term maintenance treatment, the drug has been discontinued, is through the damaging effect the drug has on mucus (Guslandi, 1980). Cimetidine causes a qualitative change in secreted mucins by reducing the amount of neutral mucins and increasing acid glyco-protein (Forstner et al.,1982).

Antibiotic interactions with mucus

Under pathological conditions the mucus produced at various sites in the body may be either too viscous or too fluid to perform its physiological functions. A number of chemical agents (drugs) have been developed in order to modify the rheological properties of mucus. Antibiotic therapy has been commonly used, both as a prophylactic and during acute exacerbations of bronchitis. The antibiotic tries, in effect to cross the

blood-bronchial barrier and this depends upon the ability of the drug to diffuse through the mucus gel. The thickened hypersecretory mucus produced during infection may present a barrier to diffusion, which is, in turn, possibly affected by the antibiotic itelf (Brown et al.,1982).

Tetracyclines produce a thickening effect on the mucus gel in the order doxycycline, tetracyline and oxytetracyline (Brown et al.,1982).

Mucus-Thinning Agents

Mucolytics and expectorants reduce the viscosity and elasticity of mucus and increase its mobility (Marriot, 1982). Amongst these are potassium iodide, used as a bronchomucotopic *in vivo*, and also various cysteine analogues which act as mucolytics by reduction of the cross linked disulfide bridges of polymeric mucus glycoprotein. Compounds used for this purpose include L-cysteine, N-acetylcysteine, cysteamine and methylcysteine. Another effective mucolytic agent is (carboxymethyl)-L-cysteine (marketed as Transbronchin) when administered by mouth (Parke, 1978). Its action results from its impairment of the incorporation of sialic acid residues into mucus which results in a reduced viscosity of the secreted product. Examples of active compounds used *in vitro* to thin mucus by disrupting disulfide bonds are dithiothreitol, 2 mercapto-ethane - sulphonate, glutathione, D and DL-penicillamine, N-acetyl-penicillamine and sodium metabisulphite (Marriot, 1982). Among the non-clinically used mucus "thinners" are numerous proteolytic enzymes, trypsin, chymotrypsin, elastase, ficin, papain and pronase. The use of deoxyribonuclease for the same purpose presumably stems from its contamination with proteolytic activity. The overall effect of these enzymes is a reduction in the

elasticity rather than the viscosity of mucus. A number of different caesium, sodium, or potassium salts have been used at moderately high concentration (0.1M) to solubilize mucus gels for experimental work. Likewise a number of detergents such as Triton X100 and SDS have also been employed to solubilise mucus (Marriot, 1982; Allen, 1981).

Mucus-Thickening Agents

Mucus gels may be thickened by the addition of other macromolecules - e.g. bovine serum albumin, IgG, IgA and DNA. This is significant in lung disease once infection occurs. Boric acid and glutaraldehyde may also be included in this group (Marriot, 1982).

Cardiological Drugs

The cardiac glycoside ouabain reduces the synthesis of respiratory tract mucus by inhibiting the transport of amino acid and sugar precursors across the plasma and intracellular membranes (Parke, 1978). On the other hand, atropine, a drug which blocks the effect of cholinergic stimuli, causes a reduction in the secretory rate of mucus.

Steriod Hormones and their Analogues

Progesterone delivered locally from an intra-uterine device exerts contraceptive activity by a thickening effect on mucus. Mid cycle mucus, which facilitates sperm transport and therefore fertilisation, can also be produced by oestrogens such as ethinyloestradiol or oestradial benzoate (Parke, 1978).

Tobacco and Alcohol

This section would be incomplete without mentioning the effects on

mucus of two popular drugs, namely tobacco and alcohol. Tobacco in the chewed form has been shown to disrupt the gastric surface epithelium causing a loss of cells and lessening the protective effect of the gastric mucus barrrier (Parke, 1978). The incidence of ulceration is higher among smokers than non smokers. It has been proposed that duodenal ulceration may be brought about by a nicotine-induced decrease in HCO_3^- secretion, which results in lower duodenal pH. (Isenberg, 1975). Alcohol, because of its lipid solubility, diffuses readily into the gastric mucosal cell and disrupts the apical plasma membrane resulting in the extrusion of mucus from the cell (Guth, 1982). The combination of aspirin, acid and alcohol was found to produce severe haemorrhage in dog stomachs.

Sites of Drug Action

The sites of action of drugs on mucus may be central (via the hypothalamus or pituitary adrenal axis), systemic (involving hormones) or topical to the mucus-producing cell. Within the cell the drugs and chemicals may act on the gene, the rough and smooth endoplasmic reticulum, the cell secretory apparatus, the lysosomes or on mucus after it is secreted.

Prostaglandins

Prostaglandins have been shown to have a powerful inhibiting effect upon gastric acid secretion (Guslandi, 1980; Johansson et al., 1980), as well as exhibiting a two-fold cytoprotective effect by increasing bicarbonate secretion (Allen et al., 1980) and stimulating that of gastric mucus (Bolton et al., 1978; Kivilaakso et al., 1979). An observed dramatic effect of prostaglandin administration is an outpouring of mucus into the gastric lumen. The prostaglandins E_2 , 16, 16 dimethyl E_2 and I_2 have all

been reported to inhibit H^+ production and secretion, whilst 16, 16-dimethyl E_2 stimulated HCO_3^- secretion as well (Garner et al., 1979; Johansson et al., 1982). Both the exogenously administered and endogenously synthesized prostaglandins can protect the gastric mucosa against a variety of injurious agents, even in the presence of acid (Guth, 1982).

Bile Salts

Bile salts have been reported to have a mucolytic effect as well as an inhibitory action on bicarbonate secretion. Sodium taurocholate forms a specified layer of mucus which is brittle and possibly easily flaked off and also inhibits HCO_3^- secretion *in vitro* (Allen et al., 1980). It is conceivable that bile salts could cause a similar change in the intact stomach and the mucus could become fractured by gastric motility, leaving the underlying mucosa exposed to acid (Ross et al., 1981). Dihydroxy bile acids reduce mucus in colonic goblet cells (Lewin et al.,1979). Reflux of duodenal contents into the stomach has been implicated in the disruption of mucosal defence and the subsequent occurrence of gastric ulcer. Sodium deoxycholate, sodium taurodeoxycholate, sodium glycocholate and lysophosphatidylcholine decrease both the viscosity and elasticity of mucus (Martin et al., 1978). This indicates a structural breakdown in mucus. Bile is also capable of producing inflammation of the superficial mucosal cells, with loss of intracellular mucus (Isenberg, 1975).

Sulphated Mucin Analogues

Sulphated mucins have been considered as anti-ulcerogenic agents since they are able to inhibit pepsin activity. Thus it has been suggested that the physiological role of sulphated gastric glycoproteins is to coat and

protect the mucosa from peptic autodigestion. (Forstner et al. 1982). The fact that human small intestinal mucins are poorly sulphated is thought to account for the vulnerability of the small intestine to peptic ulcer formation in the presence of gastric acid and pepsin. Sulphate-containing polymers such as sucralfate, a basic aluminium salt of sucrose octasulphate has become increasingly popular as research tools. Sulphoglycopeptide, a drug obtained by polysulphonation of a glycopeptide extracted from pig gastric mucosa, enhances gastric mucus coating in humans (Guslandi. 1980).

2. EXTRACTION AND PURIFICATION OF PIG GASTRIC MUCUS.

2.1. INTRODUCTION.

The pig as a model for the study of mucus in gastric ulceration.

The glycoprotein component of human gastric mucus, obtained from mucosal scrapings after surgery for resection, has been shown to be similar in structure to that of the pig (Pearson et al., 1980). Both pig and human gastric mucus glycoprotein have a molecular weight of 2x10 and consist of the same type of polymeric structure which may be cleaved into subunits of molecular weight 5x10 either by reduction of disulphide links or by pepsin proteolysis (Slomiany et al., 1972; Schrager et al., 1974; Allen et al., 1982).

Recently it was reported that a polysaccharide, of molecular weight 300,000 which has not been found in pig specimens was associated with normal human gastric mucus (Clamp et al. 1983). However, the close similarity between pig and human gastric mucus in most respects makes possible the use of pig gastric mucus as a model system for study (Allen et al., 1972; Allen, 1978). The availability of large amounts of human gastric mucus is limited for ethical reasons. Another advantage in using the pig for this particular study was the possibility of induction of ulceration by ligation of the bile duct (Arnot, 1974). There was a 100% occurrence of ulcers in the pars oesophagea of pigs which had undergone this surgical procedure. This reproducibility in ulcer formation was the main reason for our choice of the pig as a model for the study of mucus in gastric ulceration.

2.1.1. Clinical Significance of the Study.

Naturally some controversy has arisen over the extrapolation to human stomachs of results obtained when the pig was used as a model. The main anatomical difference between pig and human stomachs is the presence of the pars oesophagea in pigs. This is an area of non- keratinized squamous epithelium which is histologically oesophageal in nature and regarded as an extension of the oesophagus. The controversy referred to is important since ulceration following bile duct ligation occurred in this region.

2.1.2. The Mucus Scrapings.

The molecules responsible for the viscous and gel forming properties of mucus have been shown to be glycoproteins or mucins (Hollander, 1954), and these molecules constitute between 1% and 10% by weight of the gel. Mucus gel scraped from the mucosal surface was heavily contaminated with cell debris, food etc. (Plate 4), and was found to consist of about 60% by weight of mucus glycoprotein, the rest being mainly protein, with a little nucleic acid (Allen et al., 1982).

2.2. Methods and Materials

2.2.1. Source of pigs

Young hybrid pigs of Landrace large White stock weighing between 18 and 33 kg, were used in this study. They were supplied by commercial pig breeders from farms in the Western Cape.

Selection of pigs for each experiment was random and animals of either sex were used. However, the blood group of each pig used in the study was determined before the experiments.

2.2.2. Pre-operative management

After delivery from the farm the pigs were kept in an enclosed sty, under laboratory conditions, for a variable period. They were allowed free access to water and commercial pig food, which included either a diet of pellet "Creep meal"* or commercial pig growth and sow meal. "Creep meal" is a balanced ration of corn meal,bran, pollard, fish meal, ground nuts, sugar, salt, Vitamins A,B,C and E and trace metals, especially copper, zinc, magnesium and iron. It contains 4% fat, 18% protein and has an energy content of 3060 calories per kilogram. The main ingredients of the commercial feed are protein, fibre, calcium, phosphorus and total lysine.

Each animal received piperazine adipate pre-operatively in an attempt to eradicate round worm infestation in view of the possible association between ascaris suum larvae and ulceration (Gaafar et al, 1972).

2.2.3. Anaesthesia

Anaesthesia was induced by intravenous administration of sodium thiopentone (2-3 mg per kg body weight). A cuffed endotracheal tube was passed and anaesthesia was maintained with nitrous oxide and oxygen administered by a Magill circuit using positive pressure ventilation. The gases were warmed and humidified. The stomach was decompressed by the insertion of a large bore stomach tube, which was kept in situ throughout the operation.

2.2.4. Operative technique

Full aseptic techniques was used throughout. A catheter was inserted into the external jugular vein through an antero lateral neck incision for

*Epol (Pty)Ltd., Maitland, Cape.South Africa.

the infusion of fluids in the post operative period. Hydration was maintained post operatively by the infusion intravenously of 1 litre "maintelyte" for 12 hours. Laparotomy was performed through an upper mid-line incision, extending from the xiphi-sternum to just above the pubis. The abdomen was briefly explored and any pathology noted. The intestine was drawn out of the abdominal cavity and placed in a plastic bag to prevent heat loss and drying. Thus the approach to the stomach was facilitated.

2.2.5. Ligation and division of the common bile duct

The common bile duct is the most superficial of the three structures of the hepatic pedicle and is related to the portal vein posteriorly. It passes obliquely down to the first part of the duodenum which it reaches at a point 1.0 to 1.5 cm distal to the pylorus. The distal common bile duct was identified and isolated by blunt dissection and doubly ligated with linen sutures.

2.2.6. Insertion of gastric cannula

An area of the greater curvature of the stomach was mobilised and a 3cm incision made with cutting diathermy. Hemostasis was secured and a Thomas type purse string chromic catgut suture inserted, brought out through a separate incision on the lateral abdominal wall and secured to the skin with a dermalon suture.

Abdominal closure was the same in all operations. The parietal peritoneum and linea alba were repaired in two layers with No 1 chromic gut; the skin was repaired with dermalon.

The cannula was corked to prevent leakage of gastric juice.

2.2.7. Control (sham) operation

The sham operation consisted of the insertion of the cannula without the ligation of the bile duct.

2.2.8. Post-operative management and endoscopic assessment.

The animals were placed in holding cages for 24 hours following surgery and were allowed free access to water but no food. They were maintained on intravenous fluids throughout the study and were also given intravenous penicillin (2,5 m U).

Endoscopy began approximately 16 hours after each operation. The pigs were slung on Pavlov frames (Plate 5). The stomachs were drained of their gastric juices and rinsed with distilled water with the aid of a syringe.

The endoscope (Olympus model) was used to assess the state of the mucosa in the region of the pars oesophagea and to photograph it (see plates 6 to 10). Groups of pigs were sacrificed according to required criteria of the protocol, namely, the pre-ulcerated, ulcerated and post ulcerated state. The animals were sacrificed with intravenous pentothal and 20% potassium chloride.

2.2.9. Removal of stomach

Immediately after sacrifice, the stomach was removed. The greater curvature was mobilised by dividing the gastro-colic omentum and ligating the short gastric vessels proximally. The oesophagus was mobilised for 4cm proximal to the oesophago-gastric junction and the two vagal trunks were divided. The lesser omentum was opened and the left and right gastric vessels and accompanying branches were ligated and divided, thereby freeing the lesser curve.

The pylorus and first part of the duodenum were mobilised and non-crushing bowel clamps were placed across the duodenum and distal oesophagus. The stomach was then excised between the clamps.

2.2.10. Preparation of the stomach

All excess connective tissue around the greater and lesser curvatures was removed. The oesophagus was excised flush with the external surface of the stomach. The duodenal stump was excised distal to the tonus pyloricus. The luminal contents were drained from the stomach and collected for pH measurement.

The gastric cannula was removed carefully to avoid tissue damage. The stomach was then weighed. The stomach was opened along its lesser curvature so as to expose the mucosal surface. The four regions of the stomach were identified according to the macroscopic appearance of the mucosa (Plates 2, 3). The difference between normal and ulcerated stomachs was immediately obvious (Plates 11,12). A careful incision was made through the junction between the cardiac gland area and the rest of the stomach. Care was taken during this procedure to avoid loss of mucus. An excision of a relatively small area overlapping the pars oesophagea and the cardiac gland area was made to obtain tissue for a histological study.

The materials scraped from the mucosal surface of the cardiac gland area and pars oesophagea were drained into a pre-weighed glass vial containing cold extraction buffer (See 2.2.11). Mucus was scraped with a glass slide and pressure was applied to ensure the removal of as much mucus as possible. Immediately after scraping, the cardiac gland area was placed in a beaker of 0.9 M saline.

2.2.11. Parameters for estimation

Stomach scrapings were added to a pre-weighed vial containing 5.0 ml of 0.1M sodium acetate acetic-acid buffer, pH 5,8 (extraction buffer) (Hall et al.,1980) and 0.2 M NaCl (Robson et al., 1975; Allen, 1977). Thus the weight and the volume of the scrapings could easily be determined. The dry weights were determined by freeze drying the samples. Since the carbohydrate residues of pig gastric mucus glycoprotein include not only neutral sugars but also N-acetylhexosamines, the anthrone assay described by Seifter et al.,(1950) was found to be unsuitable for the determination of mucin concentration. Hall et al., (1980) have described a technique for the measurement of respiratory mucus glycoprotein that involved its precipitation with a cationic Alcian Blue dye. The blue precipitates were subsequently dissolved and the absorbances (at 620 nm) read to determine concentration. It was suggested that this assay method was a suitable alternative to the phenol-sulphuric, anthrone and orcinol methods,the sensitivity of which is affected by the presence of contaminants such as protein and nucleic acid. Also, these conventional methods require the use of large amounts of sulphuric acid, which constitutes a hazard. Hall et al.,(1980) claimed that their method was superior to the periodate/Schiff stain method of Mantle et al., (1978). In the present work the Alcian blue method (Hall et al.) was not reproducible, especially since the accurate estimation of the dye-glycoprotein complexes formed required large quantities of mucin: unless large amounts were used incomplete precipitation occurred. For this reason some time was spent in a establishing a reproducible system based on the PAS assay of Mantle et al (1978).

Details concerning the technique and the type of standard curve

obtained are shown in Appendix C. It was found essential in order that the technique should work reproducibly that all standard and test samples to be assayed by this technique be dialysed so as to be salt-free or contain very low levels of monovalent cations.

2.2.12. Measurement of surface area

To measure the surface area it was necessary to strip off the thin mucosal epithelial layer from the underlying mucosa. The procedure was facilitated by pinning the strips on a wax surface. The least folded epithelium strips were then sandwiched between two sheets of perspex to ensure spreading out of the mucosal tissue. A trace of the outline was made and transferred on to graph paper of 1 cm^2 units. The units enclosed by the outline were cut and weighed. The surface area was determined by relating the weights to a standard weight of a fixed number of units.

2.2.13. Extraction and purification of mucus (See appendix A for scheme)

The freeze dried mucus scrapings (for the determination of dry weights) were suspended in 25 ml of cold extraction buffer. In order to preserve the rheological properties of the mucins, and to retain them in their native undegraded form, relatively mild extraction buffers were employed. Pig gastric mucus gel was completely soluble in our solvent of choice, 0.1M sodium acetate buffer pH 5.8 buffer containing 0.2M NaCl (Robson et al. 1975), after brief homogenization for 60 sec. (Allen, 1977). Preparations of glycoprotein obtained in this manner possessed their full gel-forming properties (Allen, 1981). Precautions were also taken to avoid proteolytic degradation during the procedure. Extractions were performed

on ice and the pH of the extraction buffer was chosen (Hall et al., 1980), to minimise pepsin activity (Pearson et al, 1979). The addition of sodium azide (0.02% ww) to the extraction buffer ensured the elimination of bacterial growth.

Insoluble debris remaining after extraction was removed by centrifugation at 23000g for 10 minutes in a Beckman centrifuge and the pellets were discarded. These pellets which could be solubilized by digestion with pronase (Sigma Chemical Company), were shown to contain very little carbohydrate positive material as assessed by the PAS test, which was taken as an indication of the absence of neutral mucins. Various methods were employed to determine the carbohydrate content of our mucin preparations. These included the Alcian blue technique of Hall et al.,(1980) and the periodate/Schiff (PAS) method of Mantle et al.,(1978). The latter was preferred: the merits of these two procedures are discussed in Section 2.3.6. Supernatants were concentrated by partial freeze-drying and assayed for protein content using the Bio-Rad procedure (Bradford, 1976),(See Appendix B).

The total protein content of the various fractions obtained during the purification proedure was also determined using the Bio-Rad technique (Appendix B) and discussed under the relevant sections.

Samples of supernatants were purified by Sepharose CL-4B column chromatography, using a 100cm long x 1.6 cm I.D. column eluted at a flow rate of 30 ml/hr with 0.01 M sodium acetate buffer, pH 5.8, containing 0.2 M NaCl. Two main fractions were obtained, indicated by an excluded peak (A) and an included peak(B) in the chromatogram (see section 2.3, Fig. 4). The

fraction corresponding to peak A was further purified by caesium chloride density-gradient ultracentrifugation (Fig. 5).

The fractions obtained after the Sepharose Cl-4B step were concentrated either by partial freeze-drying or by vacuum pressure dialysis on a Pro-dicon dialysis kit. After the removal of excess water in this manner samples were added to a solution of CsCl to give a final concentration of 3.5M with respect to the latter, and were then centrifuged in 10 ml plastic centrifuge tubes, at 105000g for 48 hours at $5^{0}C$, on a Beckman L-65 ultracentrifuge. After centrifugation 1 ml fractions were collected from each tube with the aid of a two channel Gilson pump and a LKB fraction collector. Each fraction was then dialysed in the cold against distilled water to remove caesium chloride. The protein (absorbance at 280 nm) and carbohydrate contents (PAS) were determined in each case (Fig. 5), and the carbohydrate-rich fractions were pooled, concentrated and dialysed against 0.075M sodium phosphate buffer overnight. After dialysis mucin samples were finally purified by one further column chromatographic procedure using Sepharose CL-2B, (column dimensions 27 cm in length x 1.6 cm I.D., flow rate 10 ml/hr and eluted with 0.2 M NaCl).

2.3 Results and Discussion

2.3.1 Endoscopy and histology of normal and ulcerated stomachs

Macroscopic changes in the region of the pars oesophagea occurred almost always 16-19 hours after bile duct ligation. The fibre-optic gastroscope was introduced into the stomach via the gastrostomy cannula. A photographic record was made with an Olympus camera which was attached to

the lens of the gastroscope. The endoscopic appearance of a normal pars oesophagea in a sham-operated animal is shown in Plate 6. The bright yellow-bile stained pars oesophagea in contrast to the red glandular mucosa of the cardia, is clearly evident. The stage of early pre-ulceration (plates 7,8) at about 16 hours post-operatively showed signs of denudation of the squamous epithelium of the pars, as indicated by arrows. The denudation became more extensive (plate 9) about three hours later. The typical ulcer which occurred in the pars 24-27 hours after bile duct ligation showed a complete denudation of the epithelium with patches of black clot in the ulcer crater (plate 10).

Pigs were sacrificed at approximate times depending on the stage of ulceration required, and the stomachs were cut open along either the greater or lesser curvature. Plates 11 and 12 are gross views of a normal and ulcerated stomach, respectively, showing obvious difference regarding denudation of the pars and an overall bloody appearance in the latter.

The above endoscopic findings were further confirmed histologically. The normal pars cardia junction was stained both by the conventional H & E stain and Masson's Trichrome stain (Plates 13,14). In plate 13 the pars is on the left with stratified squamous epithelium, whilst the cardia is shown to have typical gastric pits and cardiac glands. In plate 14 the pars stained brown and the cardia green.

The pegs of stratified squamous epithelium projecting down into the lamina propria was the characteristic feature of the normal pars (plate 15) whilst in the normal cardiac gland area a patch of lymphoid tissue projecting through the muscularis mucosa into the submucosa was shown (plate 16).

In the pre-ulcerated state the epithelium had eroded almost to the point where the lamina propria appeared on the surface (plate 17). A well-demarcated edge of an ulcer in the pars oesophagea is evident in plate 18 (arrow), whilst in plate 19 a complete loss of epithelium revealing an ulcer base is shown. In most cases a typical cuff of stratified squamous epithelium bordered the cardiac gland area in the distal margin of the pars (Plate 20)(arrow).

2.3.2. Amounts of water in scraped material from normal and operated pigs.

There was a significant difference in the water content of the scraped material from normal as compared to ulcerated and post ulcerated pigs (Fig.8). These results correlated with the protein contents of the scraped material from each group of pigs. Bleeding through the cannula may have contributed to the high water content of the scraped material from the control animals.

2.3.3 Protein concentration under different conditions.

There was a significant increase in the total protein content of material obtained from post ulcerated as compared to that from normal pigs. Here again the samples from the 48-hour sham-operated animals behaved in a manner similar to that of ulcerated pigs (Fig.9) (Table 3). The leakage of serum albumin from the wound associated with the cannula may account for this observation. Bleeding from such a source would result in high haemoglobin content and thus a high protein content for material from 48 hour control animals. The Lowry procedure for the quantitation of protein (Lowry et al., 1951) is subject to interference by thiol reagents,

carbohydrates (Bradford, 1976), tyrosine, tryptophan and cysteine (Bonitati et al., 1969).

The Biorad assay is a highly reproducible dye-binding assay with almost no interference from carbohydrates in large quantities (Bradford, 1976), sodium chloride and 0.1% sodium dodecyl sulphate. These factors made the Biorad reagent suitable for the assay of mucus glycoproteins of carbohydrate levels of 70% and a high cysteine content in a buffer system containing 0.2M NaCl.

2.3.4 Purification and estimation of gastric mucins

Gels of large pore size have been routinely used in gel chromatography for the purification of mucus glycoprotein (Forstner et al.,1973; Gibbons, 1972). The mucins, because of their large molecular size are excluded by such gels and are well separated from contaminants having lower molecular weight, such as proteins (Snary et al.,1971; 1972). Sepharose CL-4B was the gel of choice for the first step of the purification of mucins by gel chromatography. Three main fractions were obtained by this procedure (Fig. 4), fraction A, which contained material excluded from Sepharose Cl-4B and fractions B and C, which were the included material. Material corresponding to the excluded peak A contained the mucins, which had very large molecular weights whereas degraded mucins and protein of lower molecular weight were present in the material corresponding to the included peaks (B and C). An attempt was made to purify fraction B further in order to isolate degraded mucin (Snary et al. 1971). Samples of this fraction were chromatographed on a Sephacryl S-300 column of 27 cm in

length x 1.6 cm I.D. (Fig.6) and eluted with 0,1 M sodium acetate, pH 5.8 buffer containing 0.2 M NaCl. Both the Sephacryl and the Sepharose columns were calibrated with respect to Vo and Vi using pure bovine nasal septum proteoglycan and ATP, respectively. The fraction eluted at the void volume (peak D) was anthrone-positive (absorbance read at 620 nm). This contained degraded glycoprotein and some associated proteins; the anthrone-positive material consisted mainly of degraded mucin (see Chapter 3). Two further fractions were obtained (Fig. 6), one that eluted in the void volume and was anthrone positive and a large fraction corresponding to an included peak.

One of the major disadvantages of the use of gel chromatography for the purification of gastric mucins has been the incomplete removal of non-glycoprotein material. These protein contaminants bind strongly to mucins and are isolated together with them (Creeth, 1978; Starkey et al.,1974; Allen, 1981). Mucins isolated by gel chromatography in 0.2M NaCl contain up to 20% of such contaminating proteins, which are attached to the glycoprotein by strong noncovalent interactions and thus co-elute with them from Sepharose gels (Allen, 1981). To circumvent this problem density-gradient equilibrium centrifugation in CsCl (Creeth et al., 1970; Starkey et al., 1972 and 1974; Snary et al., 1972, 1974) or CsBr (Bhaskar et al. 1981) has been found necessary. Use of this technique separated undegraded mucin from the lighter protein contaminants on the basis of their different densities, the high ionic strength of the caesium salt counteracting the strong non-covalent interactions between the macromolecules (Allen, 1981),(Fig. 5). The mucins were found in the bottom fractions of the gradients in the

region of high density whereas the contaminant proteins were in the less dense parts of the gradient.

Although pig gastric mucins have been regarded as pure after the two major purification steps described above (Allen, 1982), we subjected our mucin preparations to a further purification step namely Sepharose CL-2B chromatography (Mantle et al. 1978). The column had a bed volume of 45 ml and was eluted with sodium acetate buffer at a flow rate of 19 ml/hour (Fig.7). The large fraction eluted at the void volume contained pure mucins.

The included peak eluted obtained on the initial fractionation on Sepharose CL-4B (Fig. 4) (peak B) corresponded mainly to non-contaminant mucin proteins, previously associated with native mucin, such as serum albumin, which preponderated (see Chapter 3). Haemoglobin also occurred in high proportion of the material obtained from ulcerated pigs. Pepsin and pepsinogen were also present, in varying amounts, in material from both normal and ulcerated pigs, whilst larger amounts of proteins having very small molecular weight were evident when material from ulcerated pigs was analysed by SDS-PAGE.

The material from the included peak (peak B)(Fig. 4) from Sepharose 4B chromatograph was PAS positive in operated and normal animals, which suggested that this material consisted of degraded mucins, ranging in molecular weight from the full subunit (500 000) to much smaller species. Glycoprotein to total protein ratios of the material eluted in this peak showed significant increases from normal to post-ulcerated pigs (Fig.10,11)(Table 4). This suggests that a greater rate of degradation of

mucins occurs at the gastric mucosal barrier during the course of ulceration, thus shifting the state of dynamic equilibrium of the mucus gel to a condition where there is a marked increase in the proportion of degraded mucins. This correlated with the lower pH and higher pepsin activity found in stomachs of ulcerated animals.

The amount of undegraded mucin obtained after isopycnic centrifugation in CsCl or after purification by Sepharose CL-2B chromatography significantly decreased in material from animals in the ulcerated and post-ulcerated states as compared to that from normal animals (Fig.12,13); (Table 5). From the relevant figures it is interesting to note that in all cases the material from 24-hour sham-operated pigs (controls) behaved like the normal animals (untreated), whilst the 48-hour control animals were somewhat similar to the ulcerated and post-ulcerated pigs. This is discussed under 2.3.6.

2.3.5. Discussion

In his thesis on oesophagogastric ulceration of pigs, Arnot (1974) reported on the easy vulnerability of pigs to ulceration. Intensive pig-farming techniques have resulted in an increase in pig stress factors: environmental changes, pre-slaughter conditions, transportation, starvation for 24 hours and mixing with unfamiliar animals are some of the factors mentioned as resulting in a significant increase in gastric ulceration.

This vulnerability of pigs to stress suggests that the techniques employed in the establishment of controls in the present may have brought about changes such as those observed, especially at 48 hours. The laparotomy and the insertion of the gastric cannula as described in Section

2.2. of this chapter were obviously more traumatic than any of the other stress factors cited above. It was not unusual to observe highly inflamed and ulcerated tissue in the region of the cannula, which could explain the high total protein and water contents of the samples from the 48- hour control pig, in spite of the fact that the cannula had been inserted into the body region and the changes observed were in the cardiac gland region. It is suspected, however, that the chemical changes in mucin in control and ulcerated animals occurred throughout the stomach.

Another interesting feature of the quantitative changes of mucins from control to ulcerated animals was the large standard errors in the statistical data. It has been noted that this phenomenon is not rare in relation to biological data. For example, Elder and Hearn (1982) investigated the gastric HCO_3^- concentrations in normal and diseased human subjects and reported a range from 5 to 136 $nmol.l^{-1}$ in normal subjects and 12 nmol - 960 $nmol.l^{-1}$ in those subjects with diseased stomachs. In the present study it was found that the largest variations and standard errors were evident in determinations carried out on material from pre-ulcerated pigs, in which the first signs of observable endoscopic changes occurred.

2.4. Summary and Discussion

It is important that any study of the role of gastric mucus in the maintenance of a healthy mucosa should include both the measurement of native glycoprotein and an estimation of degradation, which to some extent is a normal physiological process. There is no doubt about the importance of the dynamic balance *in vivo* between the secretion of the mucus gel and its erosion by enzymatic action, especially by the action of pepsin in the

stomach (Allen, 1981). A tip of the balance in the favour of degradation is of pathological significance.

The quantitative studies presented here have shown that at the onset of ulceration, there is less native mucin, a higher concentration of degraded mucin and a higher total protein and water concentration. In a previous study (unpublished) it was established that ulceration was accompanied by decrease in the pH of gastric juice and a higher pepsin activity.

Endoscopic investigations during the development and formation of the ulcer indicated the presence of blood on the mucosal surface. This was also evident when the stomachs were removed after the animals were sacrificed. Although this could explain the increased protein concentration accompanying ulceration, it may only partly be the reason. The contribution of cellular and intercellular material (protein) resulting from the visible erosion of the mucosal surface and the leakage of serum into the stomach lumen, which is very probably associated with the formation of the wound, are also factors to consider in explaining the observed higher protein content. Furthermore, since mucus is an anti-bacterial and antiviral agent (Florey, 1955., Forstner, 1978), decrease in the amount present could allow colonies of bacteria to form in the ulcerated area. A possible response to this is lymphocyte infiltration, another possible contributor to the higher protein concentrations. The use of SDS-PAGE to determine the protein profiles of the scrapings from normal and ulcerated stomachs is presented in the next chapter.

Bleeding may also explain the higher H_2O content of the scrapings from the ulcerated pigs. However, other possible contributors include increased water associated with proteins, serum leakage (as confirmed by high levels of albumin; see chapter 3), a likely back diffusion of acid and a proportion of water released by the degraded mucins, which, when intact are architecturally designed to retain water through the complex arrangement of carbohydrate side-chains.

Previous workers (Pearson et al., 1981) have speculated that degraded mucins are unlikely to be found on the mucosal surface of pigs' stomachs. However, in the present study the fraction corresponding to the included peak on chromatography on CL-4B (peak B, Fig.4) has been shown to be mainly protein of small molecular weight, which is PAS positive. Although this could well be glycoprotein of small molecular weight which is present in the scrapings, the high carbohydrate content indicated by PAS assays makes this unlikely. The results of the Vi studies are as explained . carbohydrate to protein ratio (Figs.10,11). Further purification of this fraction on Sephacryl S-300 (Fig.6) gave a void-volume peak containing material which was positive to anthrone. This high molecular weight species could only be degraded mucin. However, the presence of contaminant protein associated with carbohydrate made further analysis impossible. These results were corroborated by SDS-PAGE (See Chapter 3).

The fraction eluted at the void volume of Sepharose CL-4B (peak A) (Fig. 4) also had a high carbohydrate to protein ratio.

Figure 4.

Purification of pig gastric mucus glycoprotein on Sepharose CL-4B, read at an absorbance of 280 nm.

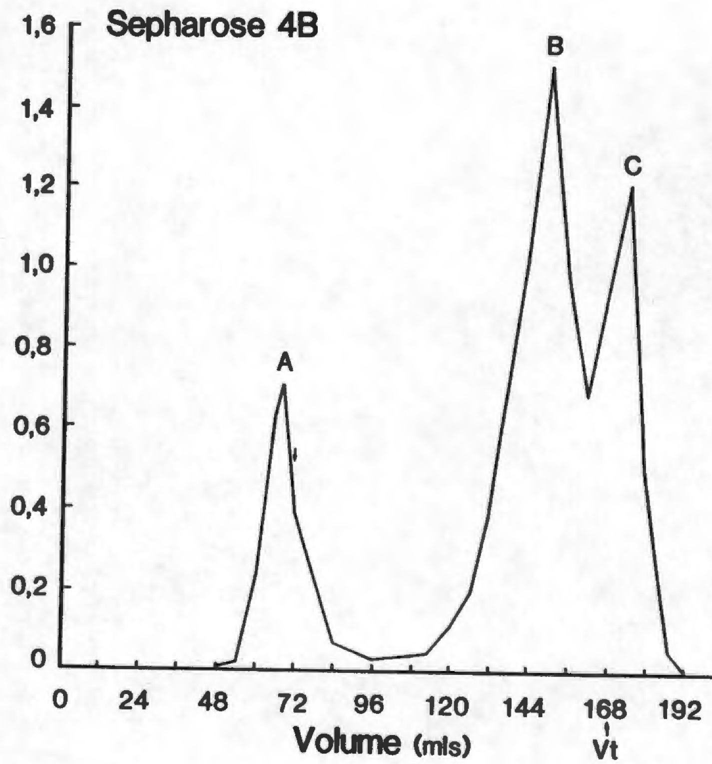


Figure 5.

Centrifugation of pig gastric glycoprotein (from peak A, Fig. 4) on CsCl density gradient.

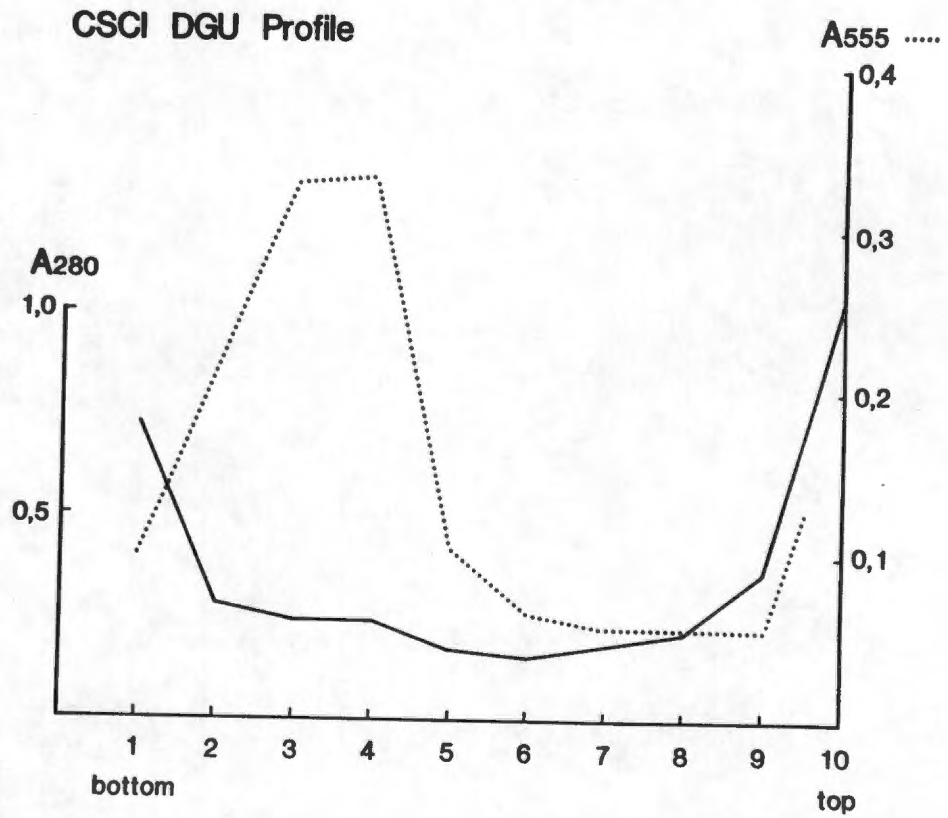


Figure 6.

Further purification of peak B (Fig. 4) on Sephacryl S-300

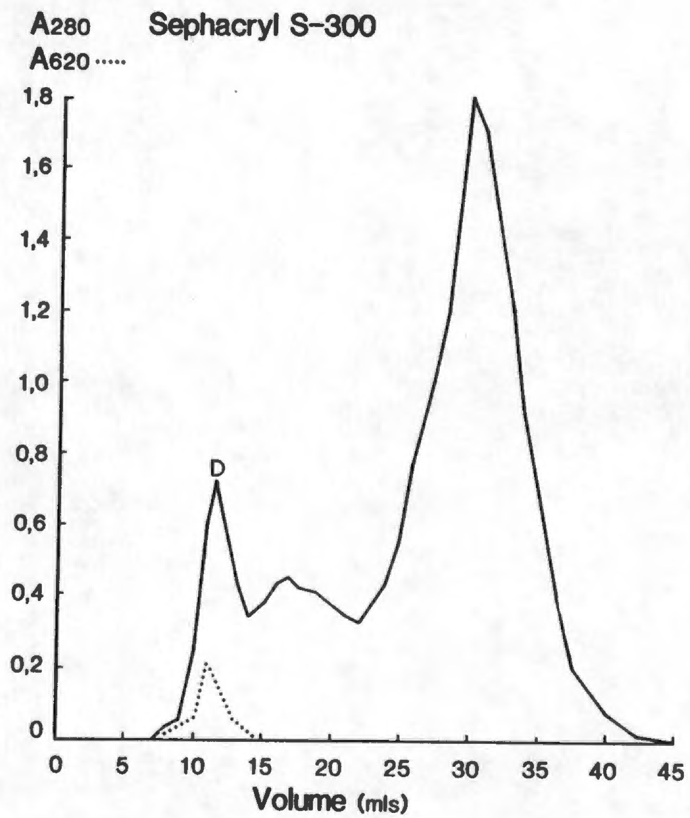


Figure 7.

Purification of pig gastric mucins for PAS positive fractions (Fig. 5) on Sepharose CL-2B

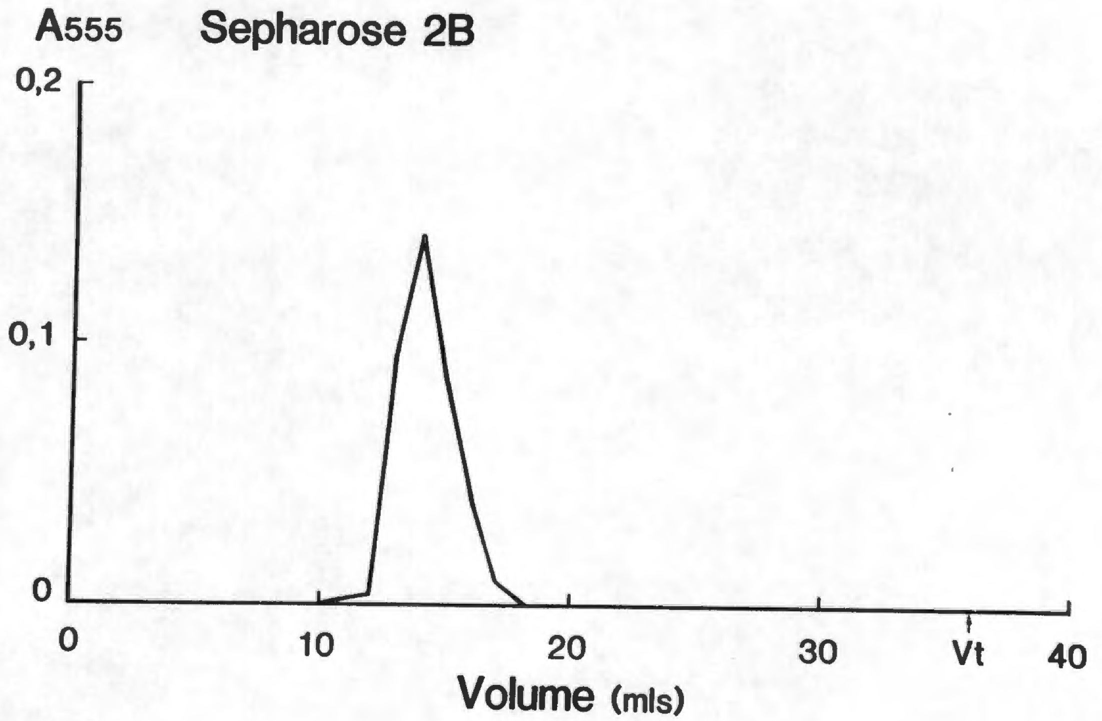


Figure 8.

The water content of gastric mucus scrapings from pigs of various conditions

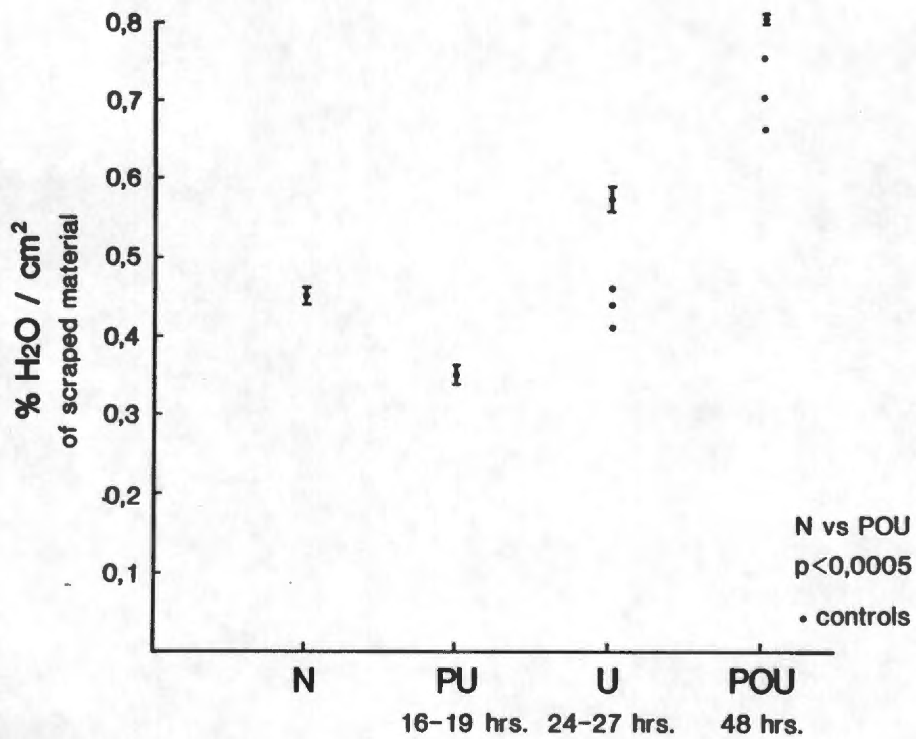


Figure 9.

The protein content of gastric mucus scrapings from pigs of various conditions

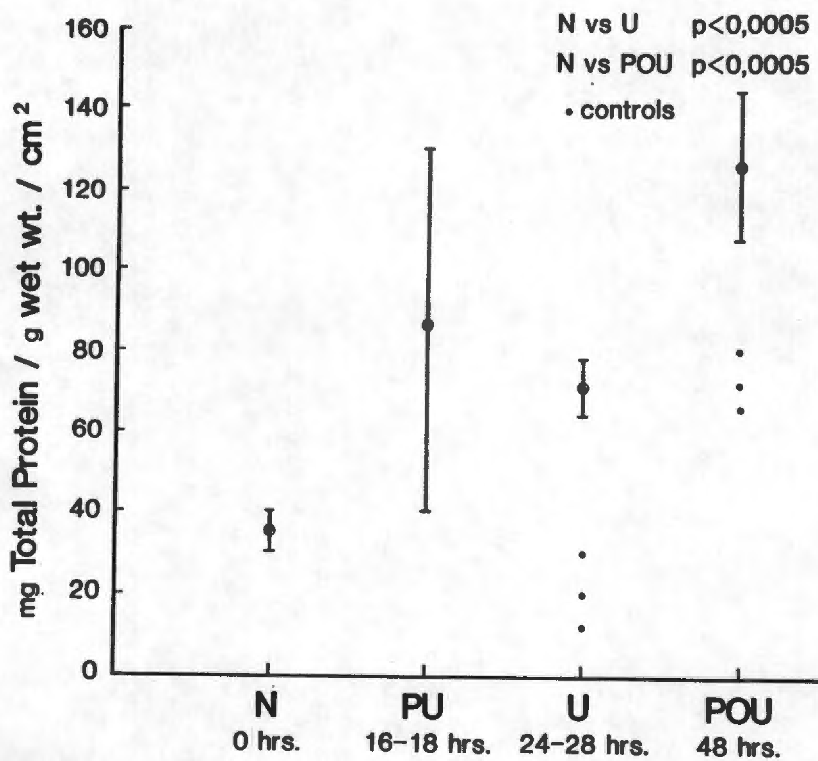


Figure 10

The degraded glycoprotein to protein ratio of fractions pooled from peak B (Fig. 4) in pigs of various conditions

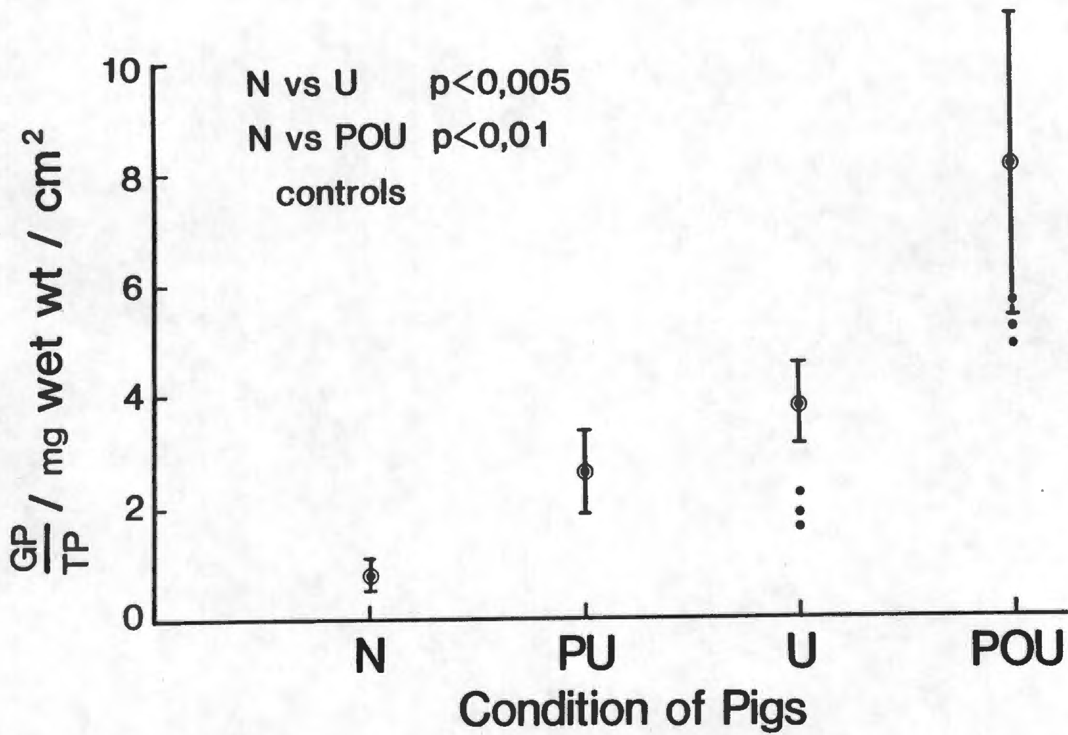


Figure 11.

The degraded glycoprotein to protein ratio of fractions pooled from peak B (Fig. 4) in pigs of various conditions

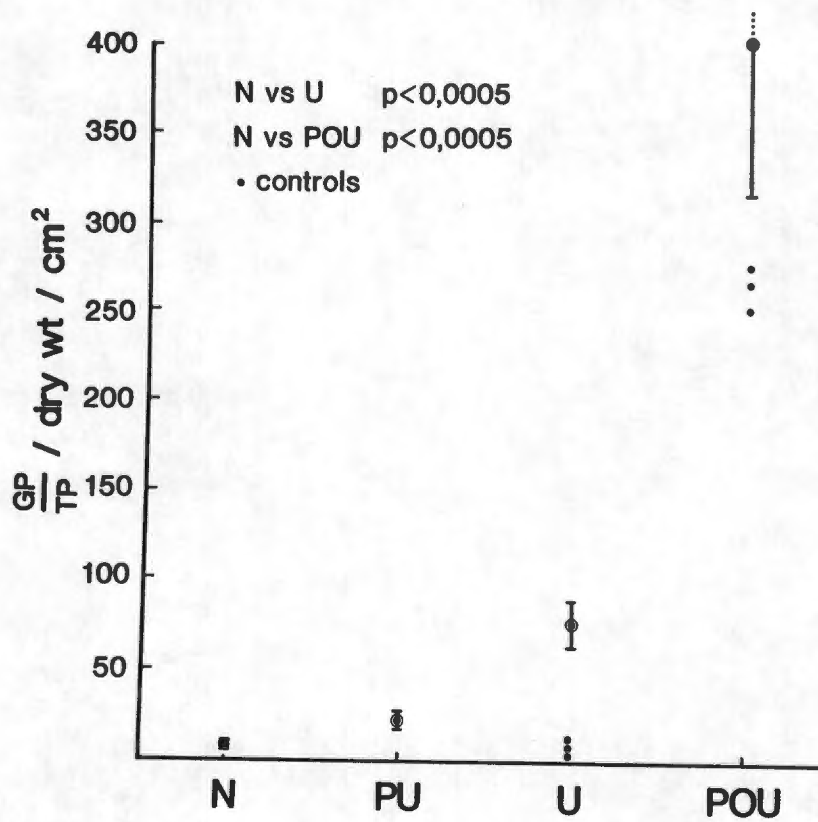


Figure 12.

The amounts of pure pig gastric mucins (pooled fractions 2-5 after CsCℓ DGU) in the various conditions

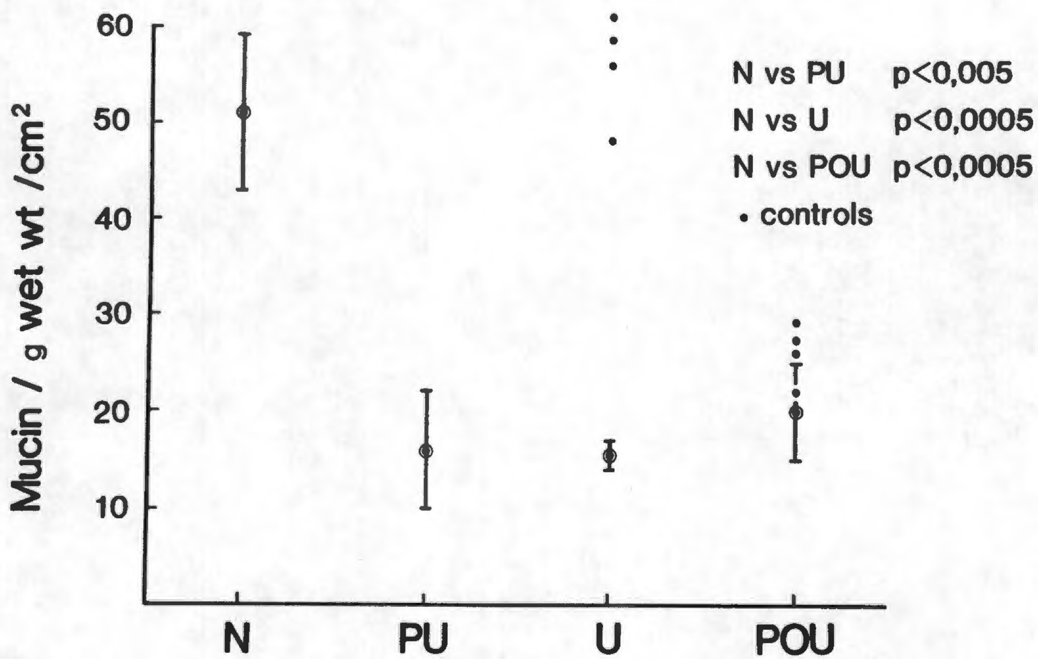


Figure 13.

The amounts of pure pig gastric mucins (pooled fractions 2-5 after CsCℓ DGU) in the various conditions

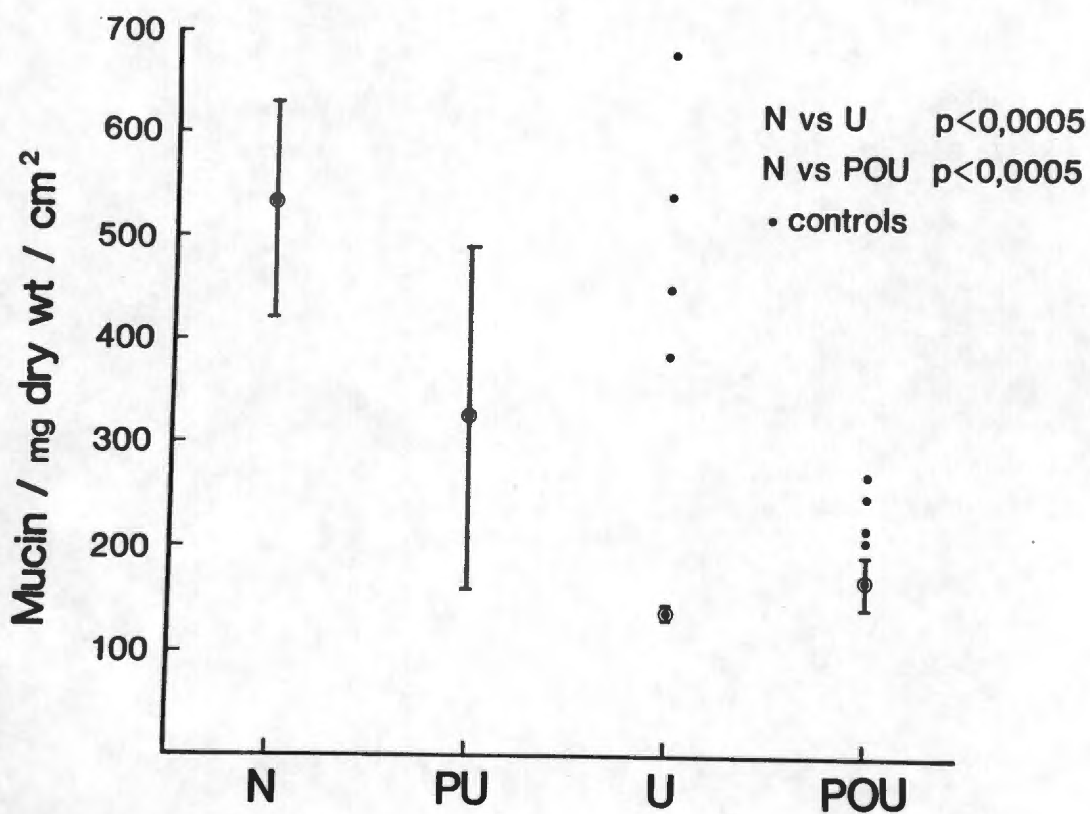


Plate 4

The constituents of pig gastric mucus.
(Allen,1981)

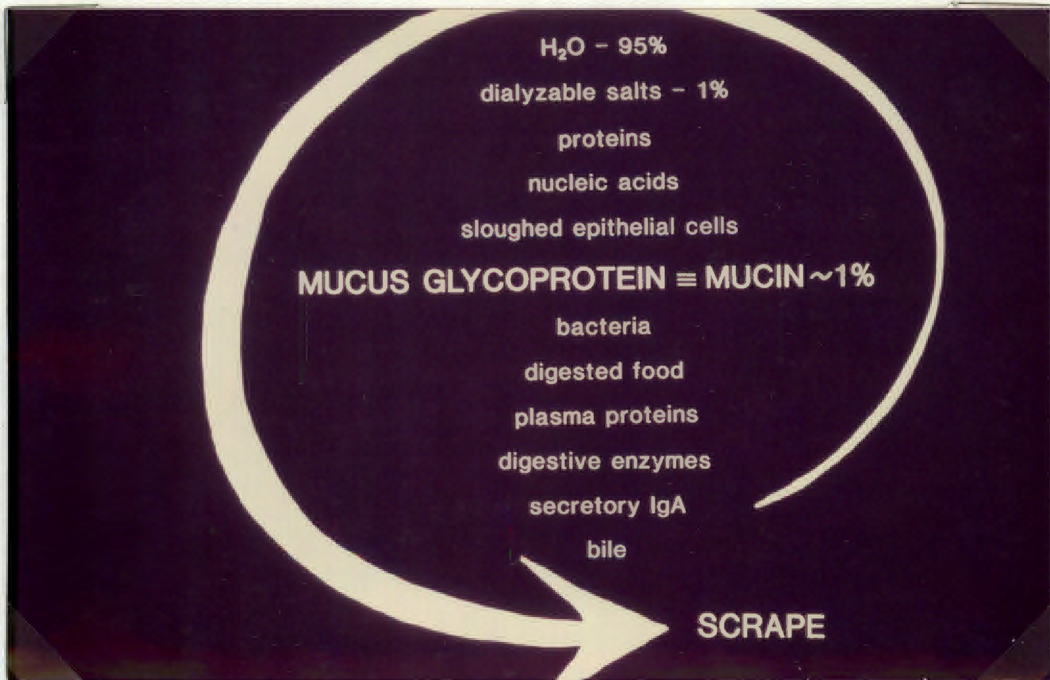


Plate 5.

A pig slung on a Pavlov frame during endocsopy.

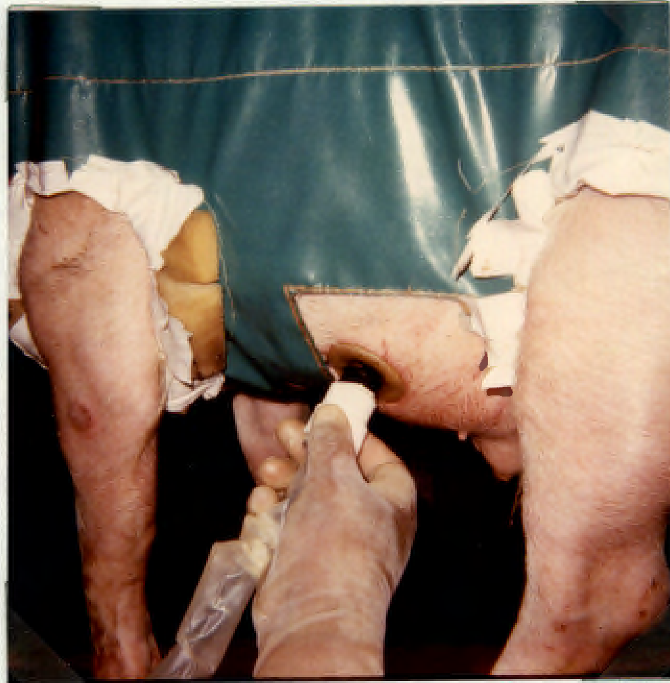


Plate 6

The normal intact pars oesophagea as seen through the endoscope

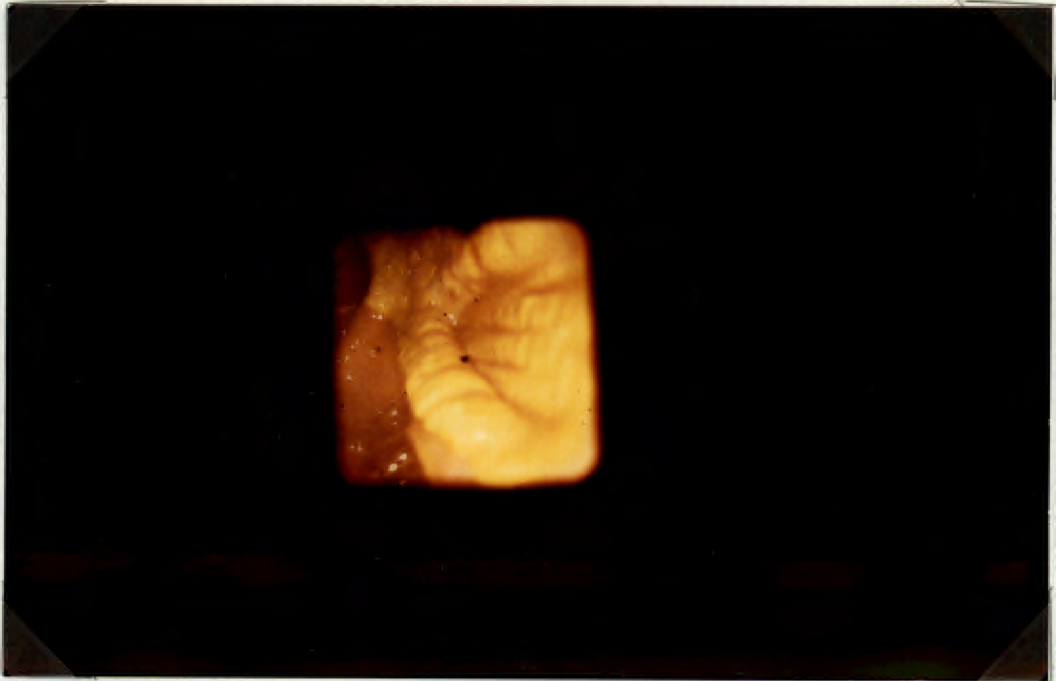


Plate 7

The first visible sign of changes (arrow) as seen through the endocope, 16 hours after the operation, the early pre-ulcer stage

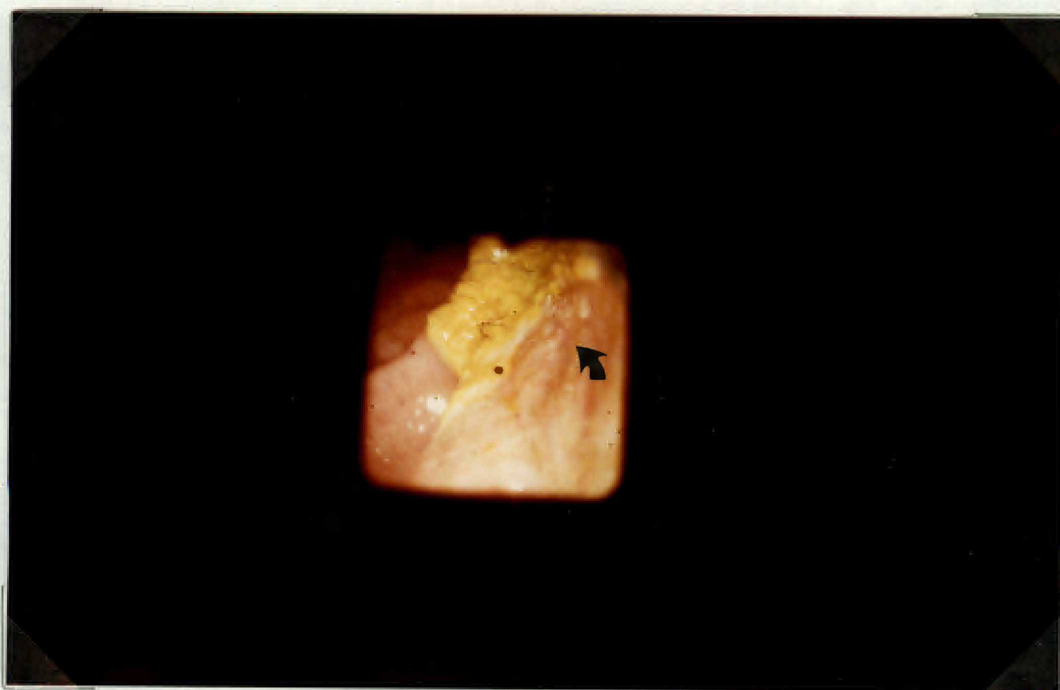


Plate 8

Denudation of portions of the pars (arrow) as seen through the endoscope at 19 hours after the operation; the early pre-ulcer stage

Plate 9

Inflammation and signs of bleeding (arrow) of the pars 21 hours after the operation; the late pre-ulcer stage

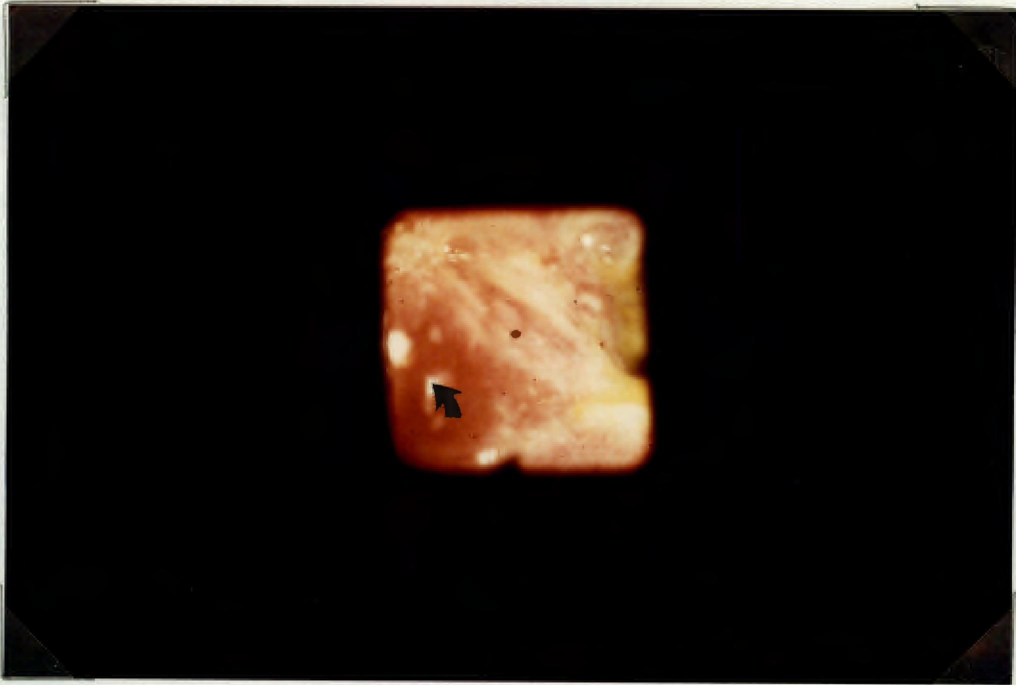


Plate 10

Bleeding and complete denudation of the pars at 24 hours;
the ulcer stage

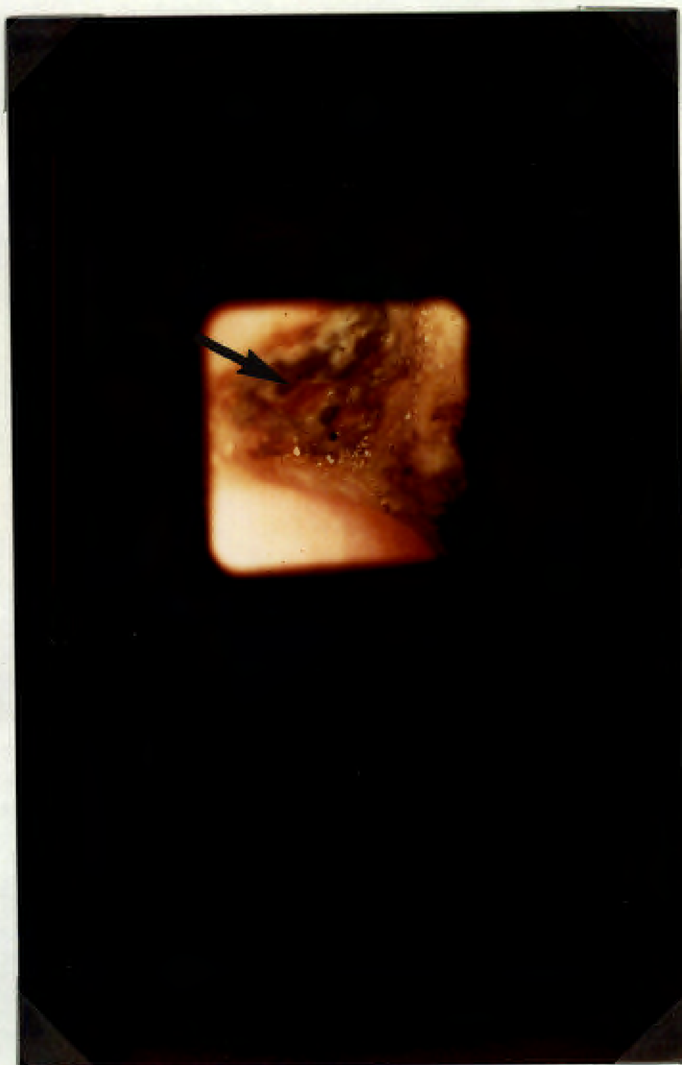


Plate 11

A macroscopic view of a normal pig stomach showing intact, bile stained pars (arrow)

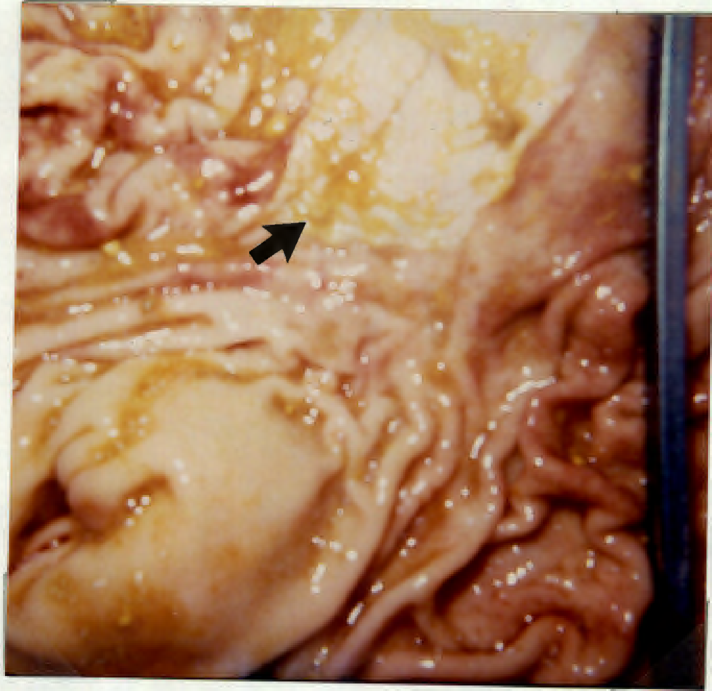


Plate 12

A macroscopic view of an ulcerated pig stomach with denuded pars (arrow) and evidence of bleeding

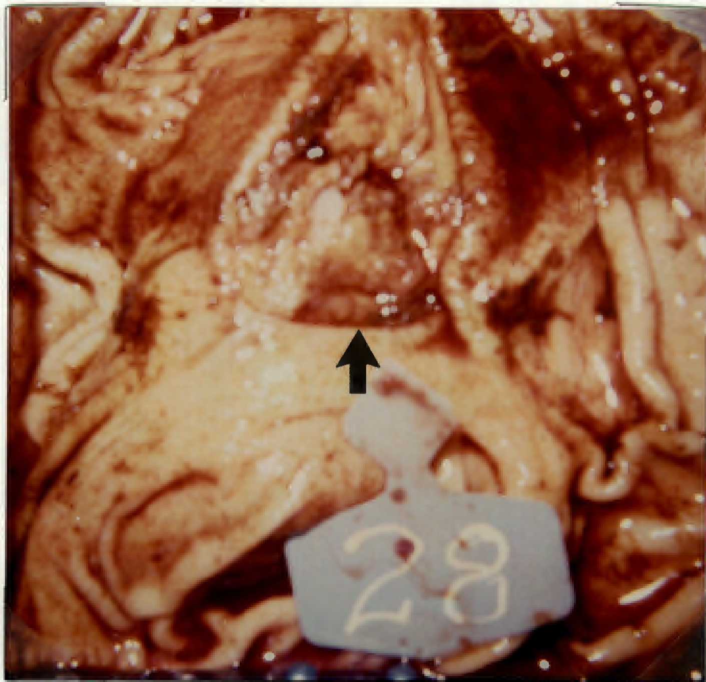


Plate 13

A histological section of the normal pars - cardia junction of the pig stomach

P = pars,

C = cardia

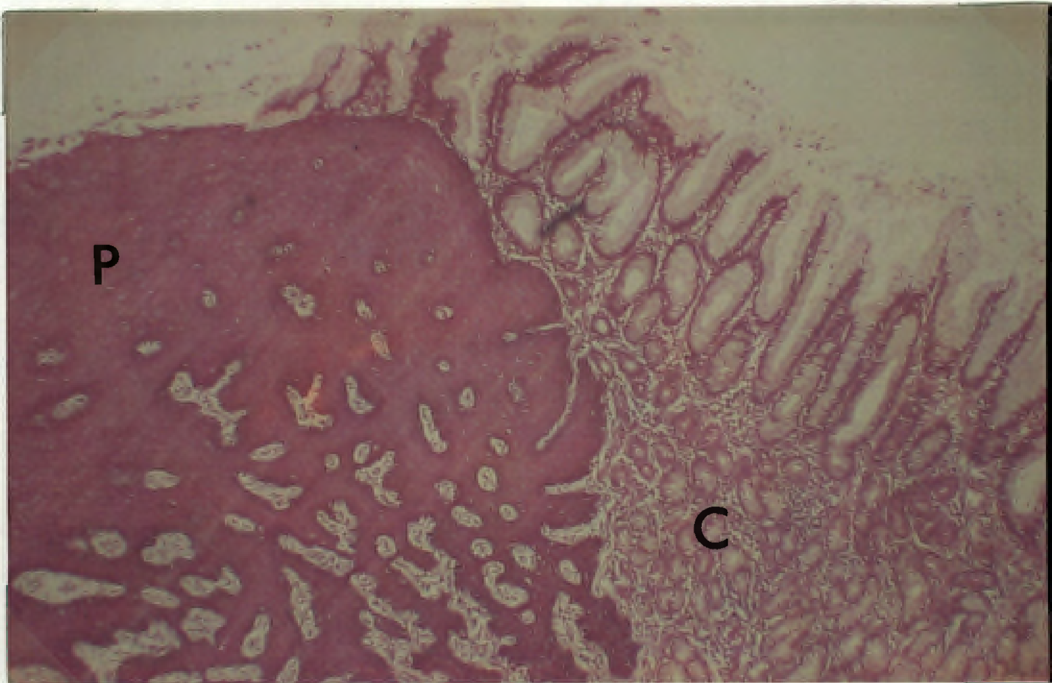


Plate 14

A histological section of the normal pars - cardia junction of the pig stomach stained differently (see text)

P = pars

C = cardia

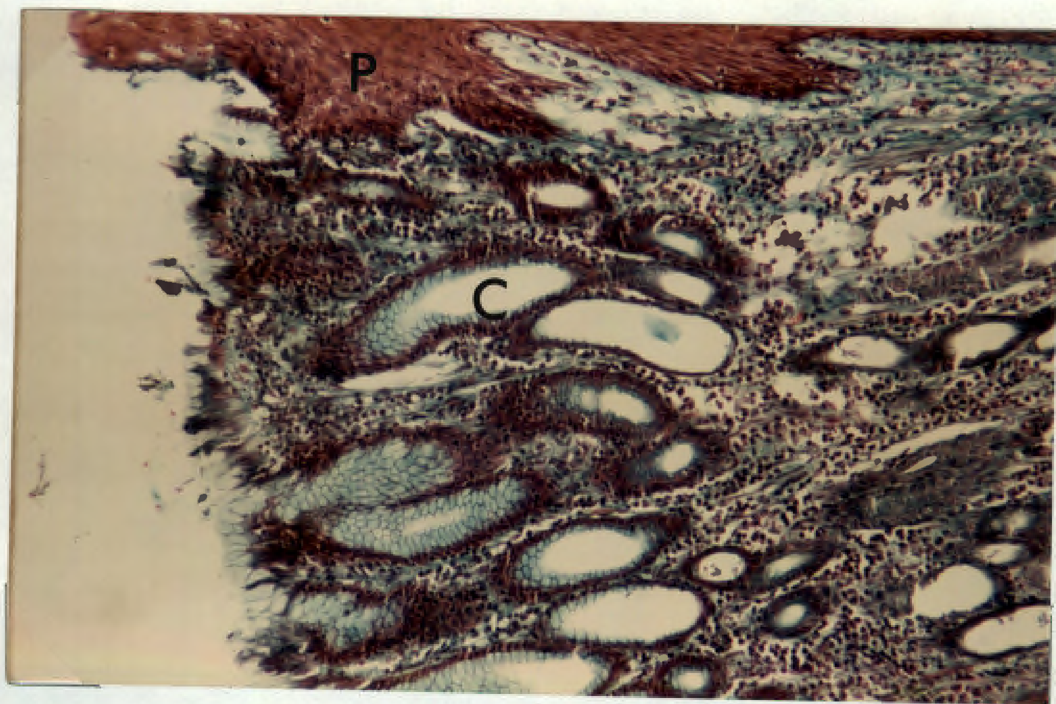


Plate 15

A histological section of the pars of normal pig stomach

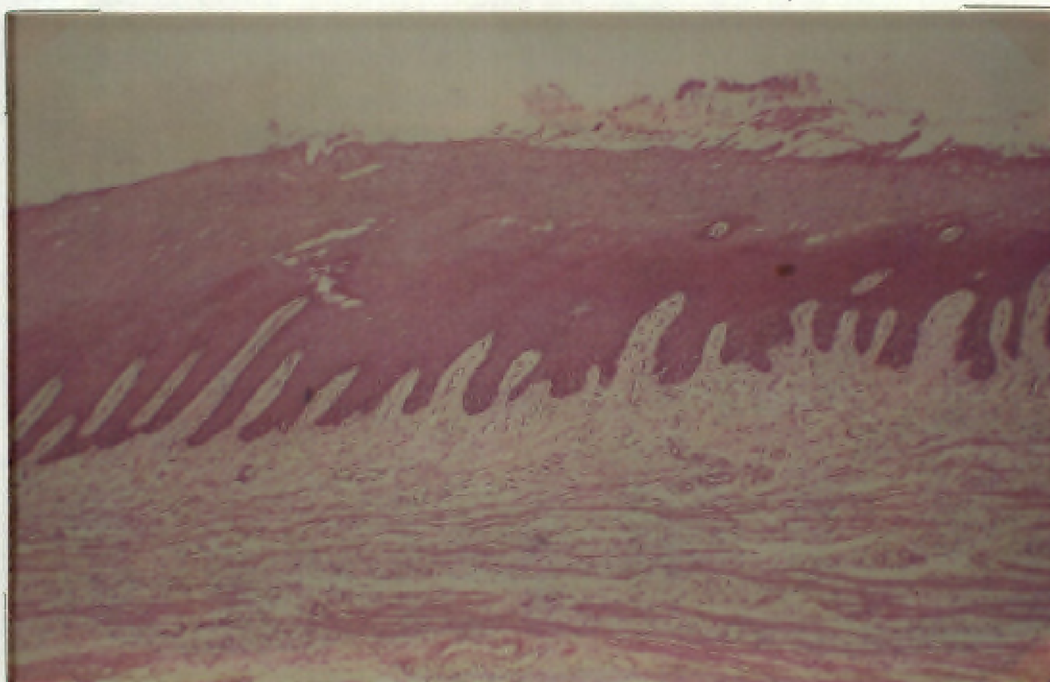


Plate 16

A histological section of the cardiac gland area in a normal pig stomach

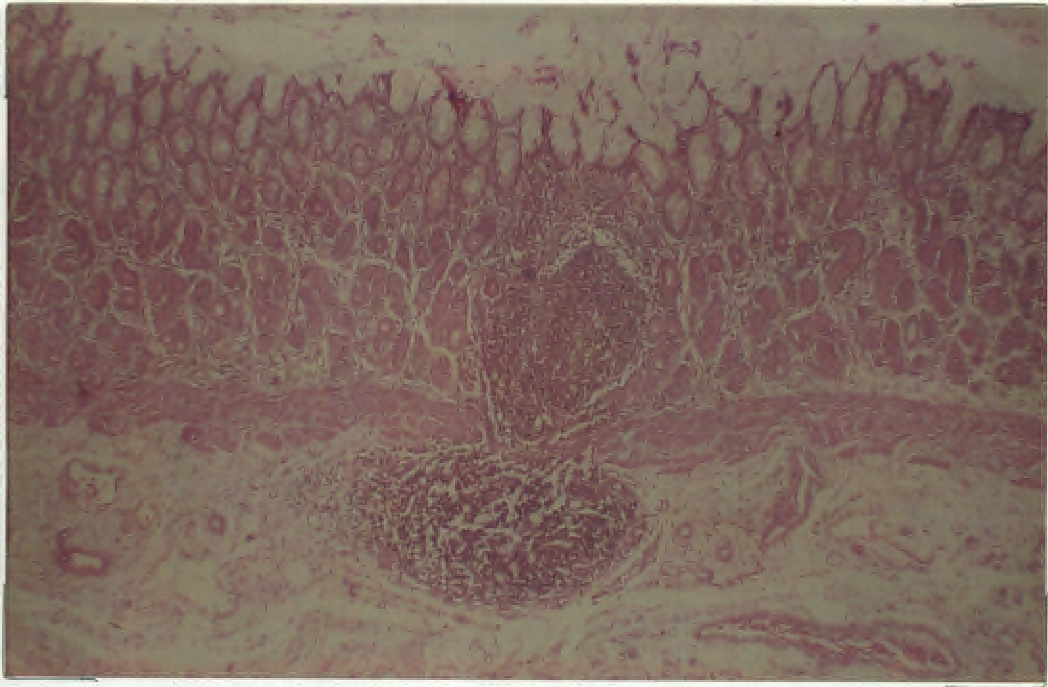


Plate 17

A histological section of the considerably denuded pars of a pig stomach during the stage of pre-ulceration

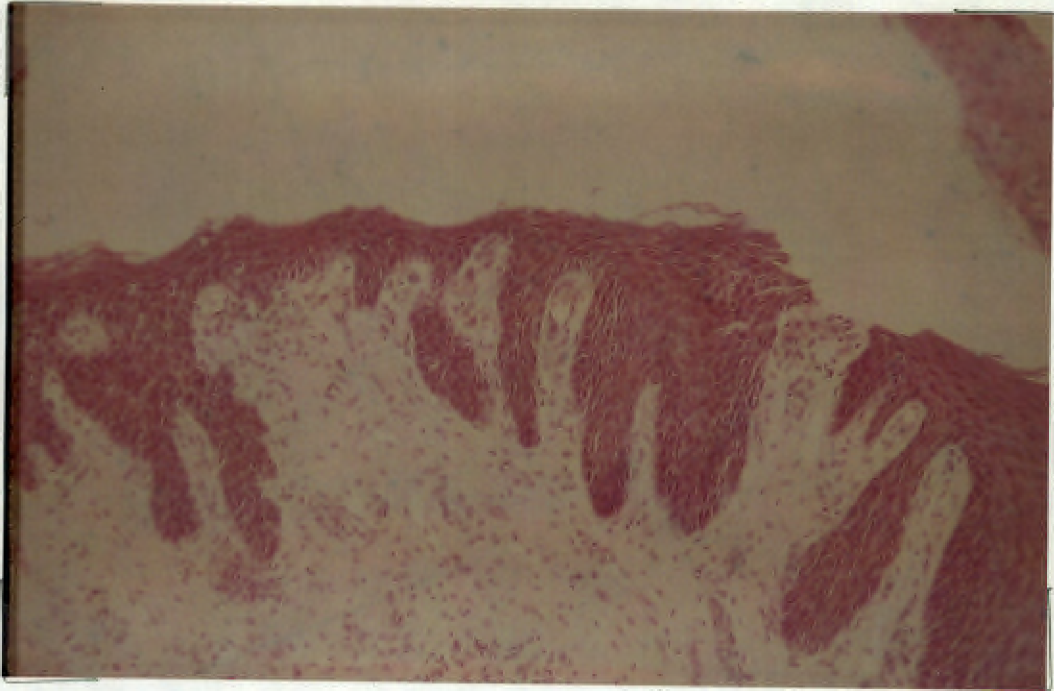


Plate 18

A histological section showing the margin of the ulcer (arrow) in the pars region of an ulcerated pig stomach

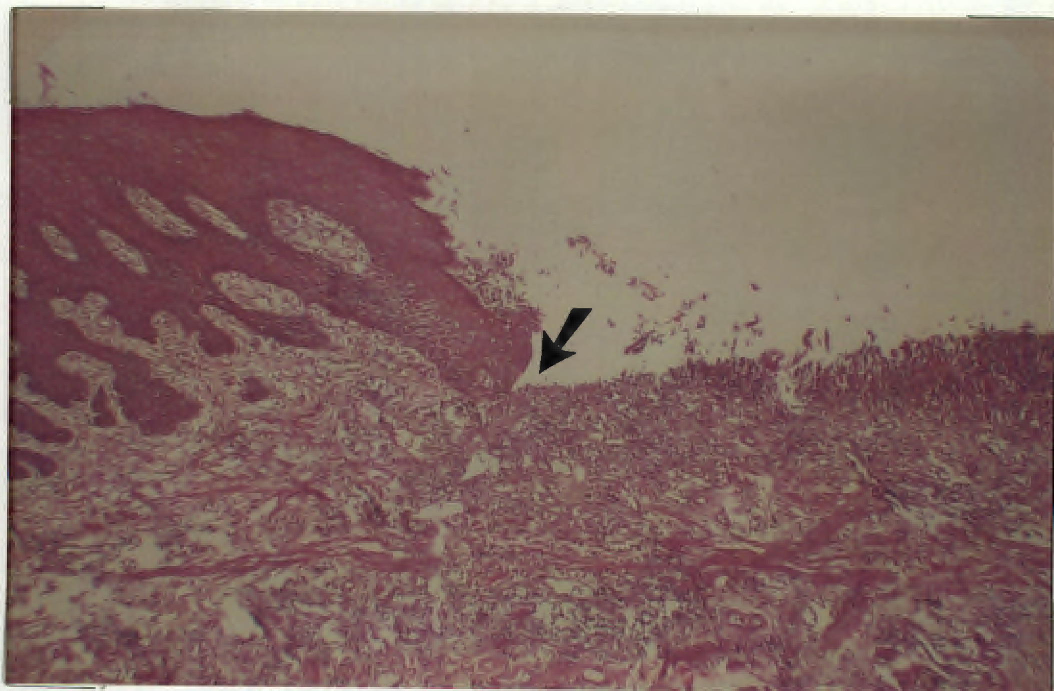


Plate 19

A histological section of an eroded pars (arrow) in an ulcerated pig stomach showing the complete loss of surface epithelium

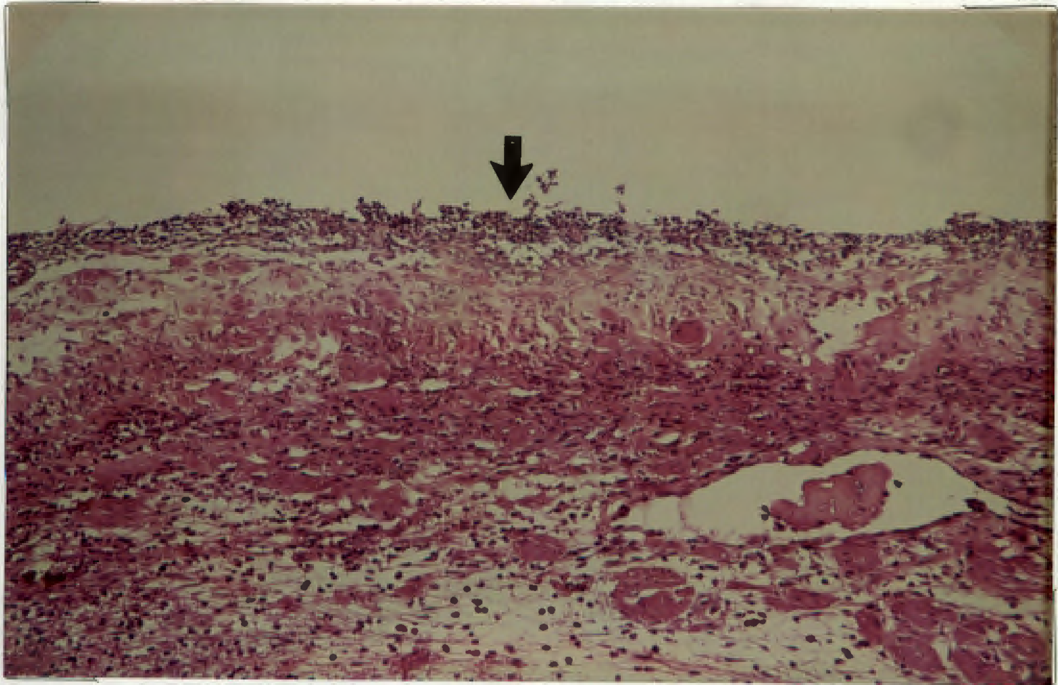


Plate 20

A histological section of the ulcerated pars cardia junction showing a typical cuff of stratified squamous epithelium (arrow) at the distal margin of the ulcer

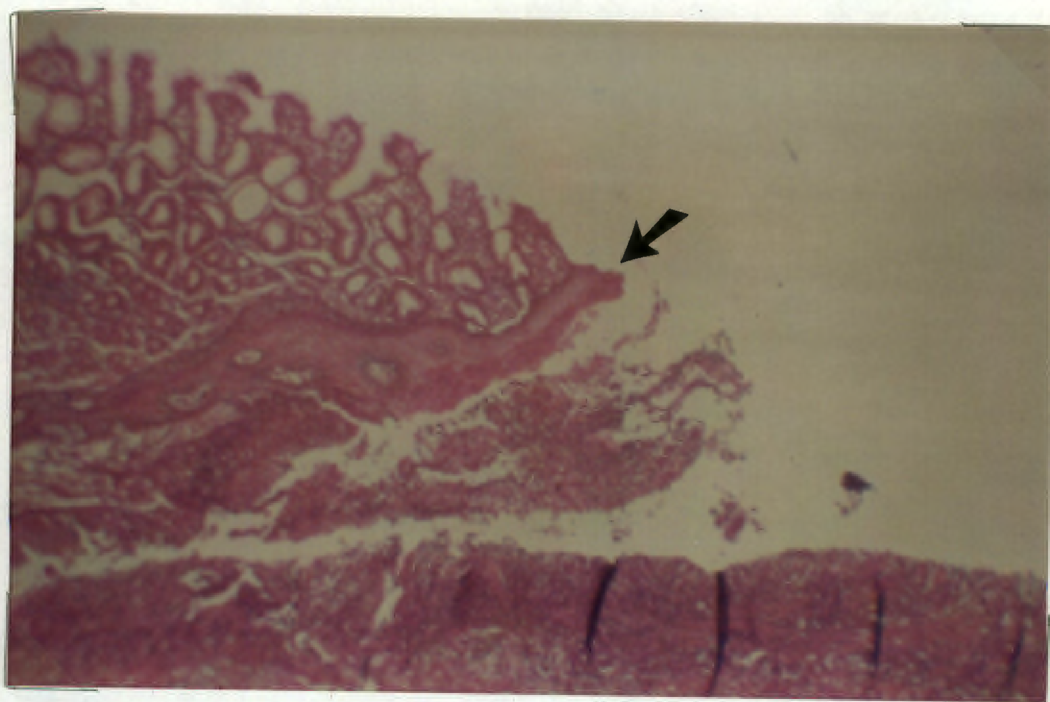


TABLE 3.

Values of total protein per wet weight and dry weights per CM^2 of normal, pre-ulcerated, ulcerated and post-ulcerated pigs.

0 hr. Normal		16-18 hr. pre-ulcerated		24-27 hr. ulcerated		48-50 hr. post-ulcerted	
ug/g ww/cm	ng/mg dry w/cm	ug/g ww/cm	ng/mg dry w/cm	ug/g ww/cm	ng/mg dry w/cm	ug/g ww/cm	ng/mg dry w/cm
25,6	150	16.87	144	83,33	160	100,74	750
49,92	380	17,74	108	75,93	750	170.89	1340
53,84	581,47	351,6	215	86,2	486	168,30	805
27,45	348,60	67,2	414	39,54	572	151,92	911
22,87	506,72	44,12	1190	82,63	750	102,74	980
35,10	500,4	21,23	440	56,85	750	54,91	470
Mean 35,80	411,20	85,63	418,5	70,7	578	124,92	876
S.D ±13,16	±154,40	±131,5	±402,2	±18,6	±233,2	±46,26	±287,43
S.E.M. 5,37	63,03	53,68	164.2	7,6	95,2	18,89	117,34

TABLE 4

Carbohydrate to protein ratio in Sepharose CL-4B included peak

0 hr Normal		16-18 hr Pre-ulcer		24-27 hr Ulcer		48-50 hr Post-ulcer	
1	2	1	2	1	2	1	2
0.3	2.7	0.59	5.0	1.8	14	7.0	660
0.41	3.4	0.027	1.6	4.7	61	2.4	460
0.76	6.6	4.4	2.7	3.7	92	10	210
0.52	8	4.0	26	1.9	23	7.0	360
1.1	14	3.9	40	5.7	71	20	130
2.0	5.1	2.8	70	4.7	95	2.4	600
0.85 ±0.63	6.6 ±4.1	2.6 ±1.9	24.2 ±27.2	3.8 ±1.6	59.3 ±34.2	8.1 ±6.52	403.3 ±210.59
S.E.M. ±0.26	S.E.M. ±1,7	S.E.M. ±0.78	S.E.M. ±11.1	S.E.M. ±0.65	S.E.M. ±14.0	S.E.M. ±2.66	S.E.M. ±85.95

N vs PU <0.025   N vs PU <0.005   N vs POU <p0.01

N vs U <p0.0005   N vs POU <p0.0005

Column 1. $\frac{GP}{TP}$/wet weight/cm^2 X 10

Column 2. $\frac{GP}{TP}$/dry weight/cm^2 X 10

TABLE 5 Values of mucin concentrations per weights and volume per cm^2 of normal control, pre-ulcerated, ulcerated and post-ulcerated pigs. (from pooled fractions 2-5 after CsCℓ DGU)

Normal cardiac gland area: Pure mucin concentrations			Ulcerated cardiac gland area: Pure mucin concentrations		
ug/g ww/cm²	ug/ml/cm²	ng/mg dry w/cm²	ug/g ww/cm²	ug/ml/cm²	ng/mg dry w/cm²
23.28 (controls) (69.1 48 hr)	23.28	202.10 (20.50 48 hr)	21.2	23.28	50.8
57.4 (54.3 48 hr)	61.29	417.4 (256.8 48 hr)	13.8	14.9	113.8
65.99 (61.1 24 hr)	67.29	512.22 (384.18 24 hr)	8.8	11.98	32.71
54.08 (47.2 24 hr)	67.59	369.11 (351.8 24 hr)	18.63	18.63	82.52
55.03 (58.2 24 hr)	53.06	828.33 (881.13 24 hr)	17.0	18.62	212.97
20.00	20.00	420.00	13.9	12.88	202.3
80.01	80.01	996.00	21.3	15.1	278.16
n=7 x̄ = 50.83 ±21.84	x̄=53.22 ±23.05	x̄ = 535.02 ±278.2	n=7 x̄=16.38 ±4.5	x̄=16.48 ±3.9	x̄=139.02 ±92.05
S.E.M. ± 8.25	S.E.M.±8.7	S.E.M. ±105.2	S.E.M.± 1.7	S.E.M.±1.47	S.E.M. ± 3.48

Pre-ulcerated cardiac gland area: Pure mucin concentrations			Post-ulcerated cardiac gland area: Pure mucin concentrations		
6.6	10.1	57.39	14.22	15.81	137.60
2.5	2.9	14.81	7.26	7.5	143.90
31.7	19.22	193.8	8.16	10.38	137.02
6.99	6.89	459.3	30.84	30.84	260.25
33.5	105.3	907.5	24.4	24.4	104.6
			34.34	34.32	250.87
n=5 x̄=16.23 ±15.03	x̄=28.9 ±43.14	x̄ = 326.56 ±368.2	n= 6 x̄=19.87 ±11.65	x̄ = 20.54 ±11.01	x̄ = 172.04 ±65.8
S.E.M. ±6.72	S.E.M.±19.3	S.E.M.±164.66	S.E.M.±4.76	S.E.M.±4.49	S.E.M. ±26.86

Statistics:			
ug/g/ww/cm²	N vs PU = $p<0,05$;	N vs U = $p<0.0005$;	N vs POU = $p<0.005$
ng/mg dry w/cm²	N vs PU not significant	N vs U = $p<0,0005$;	N vs POU = $p<0.0005$
ug/ml/cm²	N vs PU not significant	N vs U = $p<0.0005$;	N vs POU = $p<0.005$

3. CHARACTERISATION OF PIG GASTRIC MUCUS BY GEL ELECTROPHORESIS.

3.1. INTRODUCTION

Mucus glycoproteins have been difficult to characterize by the normal physical methods because of their great molecular size, poly-dispersity and high viscosity in solution. The tendency of mucins to form gels at moderate concentration and to adhere tenaciously to supporting media suggests that they are held together by intermolecular forces; this is another factor interfering with their separation and characterization by physical methods (Holden et al., 1971).

Hardly any reports appear in the literature on the investigation of mucins by SDS polyacrylamide gel electrophoresis. Problems arise with the migration of mucins in polyacrylamide gels because of their large hydrodynamic size. In 1971 Holden et al reported the use of gel electrophoresis to study the effect of physical degradation and disulfide bond cleavage on canine tracheal mucins. To facilitate entry and migration, 1-2% agarose was incorporated into their 7.5% acrylamide gel system. However, reduction of the disulphide bonds of the mucins, to depolymerise aggregates was still necessary, and furthermore, the stained protein bands were very diffuse, which is one drawback of using gels with high porosity such as those described (Holden, 1971).

Pearson et al., (1981) judged the purity of pig gastric mucins by electrophoresis in 1% SDS on 7.5% polyacrylamide disc gels, stained with Coomasie Blue; all glycoprotein preparations exhibited a strongly stained band at the origin, in the position expected for a material that had not entered the gel. Significant conclusions drawn from this investigation were: (i) that pig gastric mucins are completely pure after Sepharose 4B

gel chromatography and ultracentrifugation in CsCl; (ii) a protein having molecular weight 70,000 released on reduction of the glycoprotein is part of the covalent structure of the molecule, linked to it by disulphide bridges.

Considering the fact that pig gastric mucin consists of at least 75% carbohydrate (Allen, 1978, inter alia), we thought it essential to use a stain for the carbohydrate moiety in gel electrophoresis of the mucins. PAS has been used to stain gels for glycoproteins of the lumen erythrocyte membrane, after the gels were incubated in periodic acid. (Fairbanks et al., 1971). More recently several reports have appeared in the literature describing sensitive staining techniques for both protein (Switzer et al., 1979; Oakley et al., 1980; Sammons et al., 1981) and glycoprotein (Dubray et al., 1982). These staining processes are based upon the complexing of silver by reactive centres in the polypeptide. The procedure involves equilibrating the polypeptide-containing gel with an appropriate concentration of silver nitrate, and then placing it in a reducing solution that contains sodium hydroxide, sodium borohydride and formaldehyde. Since many of the proteins in biological fluids are present at a concentration too low to be revealed by Coomassie blue, the silver stain is the method of choice, since it is about a hundred times more sensitive than Coomassie Blue (Switzer et al., 1979).

The above procedure has also been adapted to stain 1,2-diol groups of carbohydrates, converted to aldehyde groups by oxidation with periodic acid. The method has been found to be about 60 times more sensitive than the PAS

stain, the lower limit of detection being 0.4 ng of bound carbohydrates. (Dubray et al., 1982)

However, some major problems are associated with the use of silver stains; these include the ultrasensitivity of the method which results in the production of an intense background.

3.2 Materials and Methods

The gels prepared were either 0,1% SDS - 10% acrylamide gels or 0,1% SDS, 4-20% acrylamide gradient gels. After the runs were complete, the gels were placed into the various solutions in a Pyrex tray and gently agitated on a shaker. The steps were performed at room temperature and lighting except for the periodic acid incubation of the glycoprotein-containing gels which was carried out for 1 hour at 4^{0}C (Dubray et al., 1982). During the procedure surface evaporation was prevented by covering the trays with plastic sheets.

Bromophenol blue, the tracker dye, was mixed with the buffer used in sample application. Gels were run in pairs at 50 volts and 40 milli-amperes. Under these conditions, the tracker dye took between 20 and 24 hours to reach the end of the gel. More recently it has been discovered that the gels can be run at extremely high voltages (up to 200 volts per gel) in a cold room or refrigerator (Aitken, Hendriks, Maeder personal communication), resulting in more rapid runs and improved resolution of the protein and glycoprotein bands. Protein gels were stained with 0.2% Coomassie Brilliant Blue R 250 (in 50% ethanol, 10% acetic acid in H O) for several hours. The gels were then destained with 25% ethanol, 10% acetic

acid, with frequent changes of this solution, until the background was clear and the protein bands were clearly visible.

The silver staining procedure (Sammons et al., 1981) (Appendix D) required the fixing of the proteins on the polyacrylamide gel with a mixture of 50% ethanol and 10% acetic acid, overnight. The gels were then thoroughly washed to remove SDS completely: washing involved treatment with 50% ethanol, 10% acetic acid for 2 hours, 25% ethanol, 10% acetic acid (twice for 1 hour) and 10% ethanol, 0.5% acetic acid (twice for 1 hour).

Solutions used in the staining procedure were degassed prior to use. After equilibration in silver nitrate (1.9 g/litre) for two hours, the gel was rinsed in a clean tray with degassed, glass-distilled water for 10-20 seconds, to remove excess silver and then immersed in a reducing environment. Formaldehyde (7.5 ml/litre) was added to the reducing solution immediately prior to immersion of the gels. Finally, the gel was developed in a sodium carbonate solution (7.5g/litre), which was changed at one hour intervals to remove excess sodium hydroxide. The gels were viewed on a standard fluorescent viewer.

The PAS stain for carbohydrates (Fairbanks et al., 1971) was found to be quite insensitive in the present work. The gels were therefore stained by the NH_3-$AgNO_3$technique (Oakley et al., 1980; Dubray et al., 1982) (Appendix E). After soaking in 25% isopropyl alcohol, 10% acetic acid overnight at room temperature, the gel slab was soaked in 7.5% acetic acid for 30 minutes and then stored in 0.2% aqueous periodic acid solution at 4 for one hour. The gel was washed for three hours in several changes of degassed glass-distilled water; the water was then drained off and a

freshly made ammoniacal silver solution was added to the gel. This solution was prepared as follows: (Eschenbruch et al.,1982). Aqueous NH_3 (1.75 ml) was added to 0.36% NaOH solution (21 ml) and to this solution of 20% $AgNO_3$ (4 ml) was added slowly with vigorous agitation. After the solution had cleared, glass-distilled water was added to a final volume of 100 ml. We used 200 mls of this solution to prevent sticking of the gel to the staining container. This allowed the gel to float freely. To prevent silver deposition on the surface of the gel, staining was carried out for only 30 minutes.

After removal of the gel from the ammoniacal silver stain it was rinsed in glass-distilled water. It was imperative to change containers at this stage instead of merely replacing the solution (Oakley et al., 1980). The gels were washed 3 times for 5 minutes (Eschenbruch et al., 1982) instead of once for 2 minutes (Oakley et al., 1980); this prevented the occurrence of a patchy background due to excess silver on the surfaces of both the gel and the container. A few drops of 6M hydrochloric acid were added to the washings after each change of water; after the third wash no silver chloride precipitate was formed (as reported by Eschenbruch et al., 1982). This indicated the adequate removal of excess $AgNO_3$.

The gels were transferred to a freshly prepared solution containing 0.05% citric acid and 0.02% formaldehyde (made by dilution of a 38% formaldehyde solution containing 10-15% methanol). Staining was carefully monitored at this stage since there was no guarantee of the complete absence of silver. If any deposits were noticed, trays were changed and a solution of fresh formaldehyde in citric acid was added. The gels were

stained for as long as was necessary for full development of the bands but was stopped before the background became too dark. The gel was rinsed briefly in glass-distilled H_2O and then destained in Kodafix (1:4 dilution). Farmer's reducer has also been found to be an effective destainer. After a large number of gels had been stained it was concluded that the optimal staining time was 10 minutes. The gels were then rinsed in glass-distilled water. To stop the reaction ethanolamine (150 ul) in 0.05% citric acid (200 ml) was used in the absence of methylamine, which was originally recommended (Eschenbruch et al., 1982). After 30 minutes the gels were immersed in glass-distilled water. Gels have been stored for up to 3 weeks without any effect on band intensity or resolution.

3.3. Results

Analysis by polyacrylamide SDS electrophoresis of starting material from normal, control and operated animals showed that there was more albumin (68000), hemoglobin (70000) and pepsin (34500) in the pre-ulcerated and ulcerated pigs than the normal and controls per microgram of samples applied to the gels (Plate 21, Lanes 3,4,5 and 6; Plate 22, Lanes 4-8). In this case the gels were stained with coomassie blue. Also, there was a greater amount of small molecular weight material present in the pre-ulcerated and ulcerated pigs than in the control and normal animals (Plate 21, Lanes 4 and 6; Plate 22, Lanes 6,7,8). This was more apparent when gels were stained using silver nitrate (Sammons et al. 1981) Plate 25, 10,11,12,13).

Starting material from all animals was also analysed by SDS-PAGE with staining for carbohydrate by the $AgNO_3$-NH_3 technique of Dubray et al.,(1983)

and Eschenbruch et al.,(1982). When gels stained thus were compared, the material from the 48-hour control, pre-ulcerated and ulcerated pigs showed more species of high molecular weight than did material from the 24-hour control (Plate 29, Lanes 2-5). It should be noted that the same total amount of material (ug protein) was applied to each sample well. Only trace amounts of intact mucin of very high molecular weight were evident in all samples, since this did not enter the gel and was washed away during post electrophoretic handling of gels. The high molecular weight bands observed by carbohydrate staining when samples from 48 hour control, pre-ulcerated and ulcerated animals were analysed actually represented degraded mucin molecules,, since only after partial minor degradation were the glyco-peptides small enough to enter the gel. The bands migrated with mobilities similar to those of large molecular weight fragments of commercial porcine mucin (70000)(Plate 29, Lane 1) and bovine submaxillary glycoprotein (Plate 29, Lane 15) of molecular weight 44000 (Sigma- personal communication). The concentration of fragments having the former size was greatest in the material from ulcerated pigs (Lane 5). It was also likely that there was some leakage of plasma proteins and glycoproteins into the stomachs of 48-hour control pigs, since these samples showed profiles resembling those of the samples from pre-ulcerated and ulcerated pigs, for which there was clear evidence of bleeding and thus plasma contamination of mucus (compare Plate 24, Lane 14; Plate 27, Lane 7; Plate 29, Lane 5 and Plate 30, Lane 3).

When starting material was treated with 10% mercaptoethanol (Plate 22, Lanes 10,11,12 & 14) in order to reduce any disulphide bonds present in the structure (refer Chapter 1), the bands corresponding to molecular

weight 70000 disappeared and were replaced by a number of other bands The reason for this behaviour is uncertain, but there has been some suggestion in the literature that high levels of mercaptoethanol may cause aggregation of monomeric units if treatment is carried out without a second step involving exposure to iodoacetamide.

Analysis of material that had been partially purified by Sepharose 4B chromatography (peak A, Fig. 4) gave a void volume (V_0) fraction with fewer contaminants (Plate 24, Lanes 2-5; Plate 25, Lanes 6-9), although all samples showed evidence of considerable contamination by albumin, which was most clearly seen in gels stained with $AgNO_3$ (Plate 25). When V_0 samples from the variously treated animals were incubated with mercaptoethanol prior to electrophoresis (Plate 25, Lanes 2,4 and 5), two prominent bands at molecular weights 70000 and 90000, appeared; again there was more positively stained material from pre-ulcerated than from control animals.

On gels stained for carbohydrate, the Sepharose 4B void volume samples exhibited fragments ranging in molecular weight from 40000 to 50000 (Plate 29, Lanes 6-9) and these were more pronounced in material from ulcerated than from control animals. There were also bands at molecular weights 30000 to 40000, which were progressively more evident in the 48-hour control, pre-ulcerated and ulcerated animals as opposed to 24-hour control samples (Plate 29, Lane 6).

When the same void volume samples were treated with 10% mercapto-ethanol two major changes occurred. Firstly,there was an increase in the amount of stained material at the spacer/separating gel interface (molecular weight $5x10^5$) and, secondly, the band prominent prior to

treatment with mercaptoethanol disappeared. However, material from the ulcerated pigs than controls still showed a higher number of fragments than did that from controls.

The material associated with the included peak obtained on Sepharose 4B gel chromatography (Fig. 4) was also analysed by the gel system discussed above (Plate 23, Lanes 2,3 and 4; Plate 24, Lanes 9-13). All samples, irrespective of source showed evidence of containing large quantitites of serum albumin, (Plate 23, Lane 3; Plate 24, Lanes 9-13), with the greatest amounts in the samples from the ulcerated and post ulcerated animals.

There was evidence for the presence of haemoglobin and pepsin in most of the samples derived from both control and ulcerated animals. Samples from ulcerated animals were shown to have a higher concentration of pepsin than those from control animals and numerous low molecular weight polypeptides that were concomitant with a high degree of protein degradation (Plate 23, Lanes 3 and 4). These were not so evident in samples from control animals. From these experiments it was also noted that samples from the ulcerated animals gave rise to more material that first entered the separating gel than did those from control samples. Since in this instance the analysis was carried out using a final gel concentration of 10% weight/volume, the molecular weight expected for such a fragment would be of the order of 240,000. This suggests, again, that partially degraded mucin fragments are more evident in samples from ulcerated animals. The overall profiles for material from ulcerated and post-ulcerated animals were similar and had many features in common with the gel profile for pig

serum (Plate 23, Lanes 3,4 and 7), which is consistent with the crucial observation of bleeding and thus leakage of serum components under ulcerated conditions.

When the gels were stained for carbohydrate (Plate 29) material from peak B (Fig.4) obtained on Sepharose 4B showed differences between control and ulcerated states (Lanes 13 and 14). The data supported the quantitative data presented in Chapter 2. The concentration of glycoprotein fragments which stained both for protein (Plate 23, Lanes 2,3) and carbohydrate (Plate 29, Lanes 13 and 14) was clearly higher in the ulcerated state than in the control samples. When the material from peak B was further purified on Sephacryl S-300 (Fig.6) and the void volume fraction was applied to 10% SDS-PAGE, the only difference that resulted was a decrease in the intensity of the albumin band.

Material pooled after Sepharose 4B chromatography was subjected to CsCl density-gradient centrifugation and the gradients were fractionated (see Chapter 2). After preparation and processing as described in Chapter 2, gradient fractions were analysed by SDS-PAGE (Plate 27). The proteins in the fractions of low density were found to consist mainly of serum albumin, with some evidence of polypeptides having a size consistent with that found for pepsin in material derived from ulcerated and post-ulcerated animals when this material was run and stained with Coomasie Blue (Plate 26).

Proteins in the low density fractions from gradients after ultra-centrifugation were previously associated with the higher density native mucins prior to gradient formation. It was found that these low-density

fractions did not consist exclusively of protein but also contained glycoproteins of both low and high molecular weight; the appearance of these in the low-density fractions was possibly due to carbohydrate components. There was an increasing number and intensity of these fragments from the 48-hour control pigs to the post-ulcerated animals (Plate 30, Lanes 4-9). Bands corresponding to molecular weights of $4\text{-}5\times10^5$ were more prominent for the samples from the 48-hour control, pre-ulcerated and ulcerated states (Plate 30, Lanes 5-7) as were those at molecular weight 90,000 (Lanes 9-7) and smaller fragments having molecular weights from 10000 to 90000 (Lanes 4-7). This was a clear indication of the presence of more degraded products in the scrapings from the 48-hour control, pre-ulcerated and post-ulcerated states. The presence of a greater amount of stained material at the origin in Plate 30, Lanes 4-6 implied that these mucins could enter the gel, possibly because they were smaller than normal mucins.

Pure mucins obtained after CsCl density-gradient ultracentrifugation, either alone or followed by Sepharose 2B gel chromatography, showed on SDS-PAGE the stained band at the origin indicative of pure intact mucin. This was found for all samples (i.e. from normals, controls and ulcerated pigs); (Plate 28, Lanes 2-6 and Plate 30,Lanes 10-14). When the amount of material applied to the gels was increased, material from the pre-ulcerated, ulcerated and post-ulcerated animals (Plate 28, Lanes 4,5, & 6) showed evidence of a considerable concentration of large polypeptides from degraded mucin having a molecular weight in the region of 500,000. The sample from the post-ulcerated pig gave rise to a band at a molecular weight about 70,000 (Plate 28, Lane 6) and bands at molecular weights between 40,000 and 50,000 (Plate 28, Lanes 5 & 6) were found for material

from both ulcerated and post-ulcerated pigs. These same samples exhibited prominent bands at molecular weights between 10,000 and 20,000.

When material purified by density-gradient centrifugation and Sepharose 2B chromatography or density-gradient centrifugation alone, was analysed on gels after treatment with mercaptoethanol (Plate 28, Lanes 9-11 and Plate 31, Lanes 2-5) more material migrated into the gel, which suggested that there was some degradation of intact mucin whilst in its aggregated form. The data showed that pre-ulcerated and ulcerated animals gave rise to more material that was able to migrate into the gel than did the controls (Plate 28, Lanes 9-11). A prominent fraction of low molecular weight that was found in the samples from ulcerated pigs (Plate 28,Lane 5) was also present in those from post-ulcerated pigs (Plate 31,Lanes 2 & 3).

3.4 DISCUSSION

Analysis by SDS-PAGE of the material from which the purified gastric mucins were subsequently isolated showed a high concentration of polypeptides derived from plasma, presumably due to bleeding; this was so in all cases, although only trace amounts were present in samples from control and normal animals (compare plate 24, Lane 14; Plate 27, Lane 7; Plate 29, Lane 5 and Plate 30, Lane 3). There was evidence also for pepsin-sized polypeptides and others of lower molecular weight. Thus, the main conclusions that could be drawn about "starting material" were that it contained a diversity of proteinaceous species many of which gave a positive stain for glycoprotein, and that, except in the case of normal and control animals, the bulk of this material must have been derived from plasma.

Once the material from each animal source had been chromatographed on Sepharose CL-4B there was some clarification as to the polypeptides present Thus, in the material that eluted at the void volume of the column there was evidence of glycoprotein fragments of large molecular weight that had been only just included in the fraction (because of overlap at high concentrations) and a number of fragments of small molecular weight, in samples from all animals (Plate 24, Lanes 2-5). However, material from ulcerated and post-ulcerated animals was generally shown to contain more material that had migrated into and partially through gels. Since intact mucus glycoprotein would not enter the gel at all and would therefore be "washed away" at the end of the electrophoretic run, during the staining process, the quantity of material that was in any degree included in the gel is an indication of the amount of mucin that had been degraded, albeit only slightly. It should be noted that the sample mass was kept constant in all of the electrophoretic analyses.

For material passed from the included Sepharose CL-4B column, by SDS-PAGE indicated a high proportion of serum albumin, haemoglobin and a number of small polypeptides probably derived from plasma. It was interesting to note that samples from ulcerated and post-ulcerated animals exhibited more intense bands of peptide species due to small molecular weight than did those from control or normal animals when gels were stained for glycoprotein (plate 29, Lane 6-10).

Further purification of material which eluted at V_0 from the Sepharose CL-4B column was achieved by CsCl isopycnic gradient centrifugation. On analysis of the different gradient fractions by SDS-PAGE a considerable

amount of glycoprotein that was presumably of mucin origin was found to be present in the low density fractions obtained after centrifugation of material from ulcerated and post-ulcerated animals (Plate 30, Lanes 4-9). The data suggested that a quantity of carbohydrate had been removed from this glycoprotein, thus rendering it less dense. The results presented in chapter 4 corroborate this suggestion, which may also account for the observation that intact mucin "aggregate" seems to have been truncated by protease activity in the case of ulcerated or post-ulcerated animals. This may, perhaps, indicate that some of the branched carbohydrate chains had been very largely removed at a single locus, thus rendering that site proteolytically sensitive. As yet there is no evidence for such an occurrence.

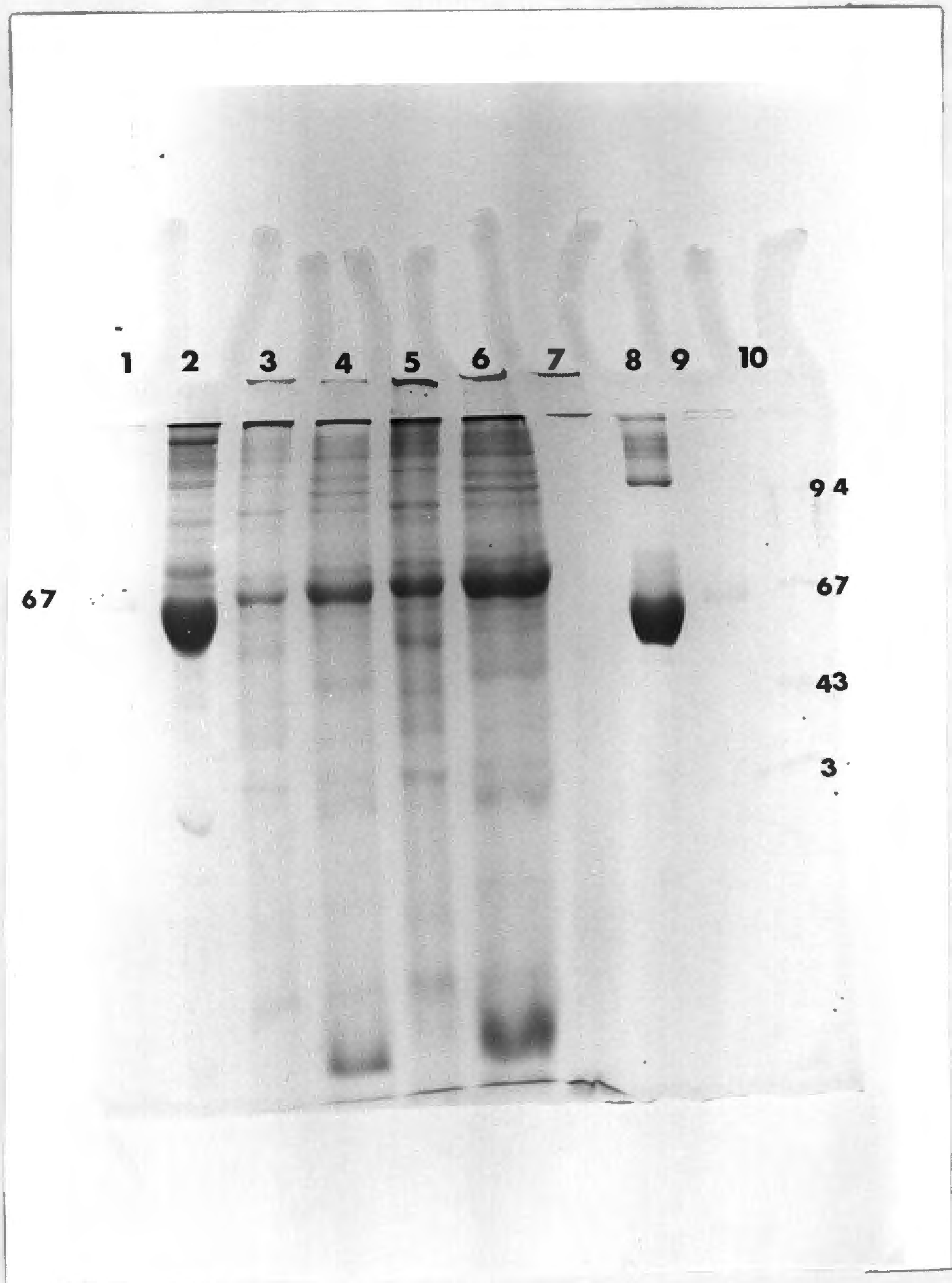
SDS-PAGE of material submitted to a final purification step by Sepharose CL-2B chromatography also revealed a number of polypeptides that migrated with mobilities indicating molecular weights consistent with those expected for glycoprotein subunits that were partially degraded; these were associated mainly with material from the ulcerated and post-ulcerated animals (Plate 30, Lanes 10-14). The fact that these sedimented on CsCl density-gradient centrifugation to a position that was consistent with their having a normal carbohydrate content suggested that degradation on ulceration may follow either of two courses, namely, one involving carbohydrases as mentioned above and one involving proteolytic digestion, presumably via pepsin.

Finally, the data illustrate the value of the new staining methods using silver nitrate, as opposed to the older, well-established Coomassie

blue staining method. For example, if the material from ulcerated and post-ulcerated pigs was, after purification by Sepharose CL-2B chromatography, electrophoresed and stained with the latter reagent no fractions of low molecular weight were evident on the gels. However, if the same samples at the same quantities were rerun and stained using the silver nitrate system (Sammons, 1982) bands at molecular weights 70,000 and 10-20,000 as well as others at higher molecular weight were visualized. The species having molecular weight 70000 may be serum albumin or the polypeptide described by Pearson et al., (1981) that "binds" the mucin tetrameric molecule together (plates 26, 27, Lanes 2-3).

Plate 21

0.1% SDS 10% PAGE

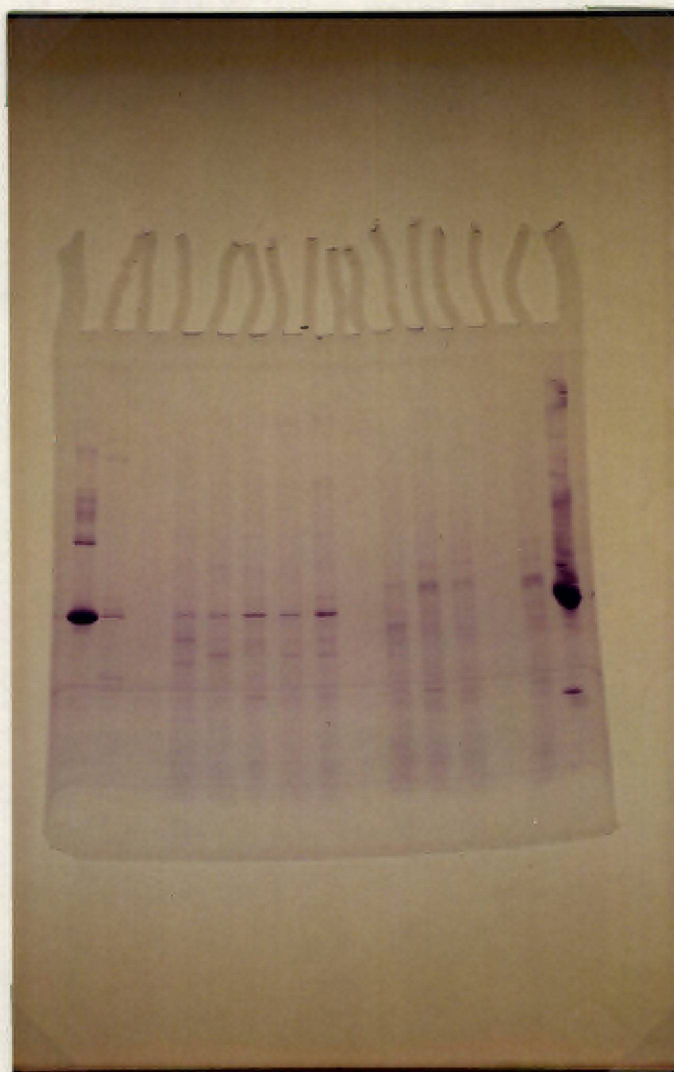


Slot No.

1. MW STD. (X10)
2. Pig Plasma
3. N 50 g sm
4. U 50 g sm
5. N 100 g sm
6. U 100 g sm
7. Pig gastric mucin (Sigma)
8. HSA
9. MW Std. (X10)
10. MW Std. (X10)

Plate 22

0.1% SDS 4 to 20% gradient PAGE

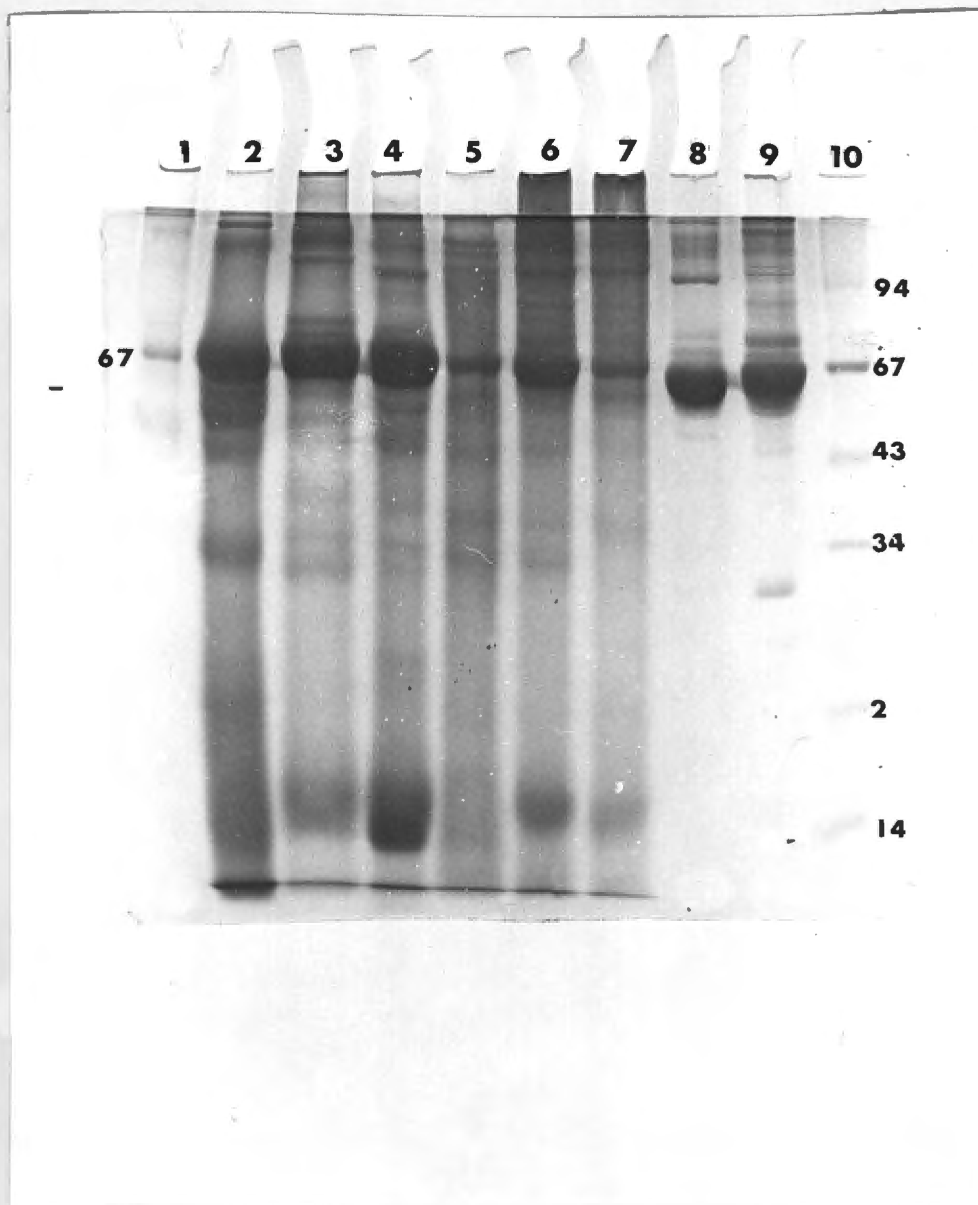


Slot No.

1. BSA	2. MW Std. (X10)	3. -
4. N sm	5. 24 hr C sm	6. 48 hr C sm
7. PU sm	8. U sm	9. -
10. N sm with MSH	11. 48 hr C sm with MSH	12. PU sm with MSH
13. -	14. U sm with MSH	15. Pig plasma

Plate 23

0.1% SDS - 10% PAGE

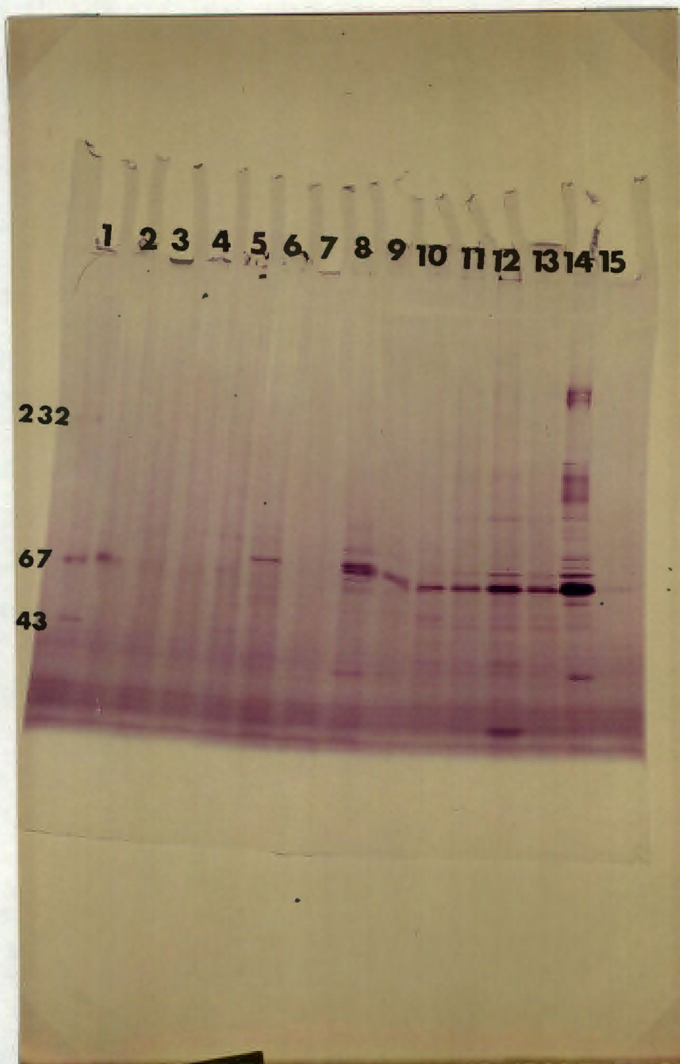


Slot No.

1. MW Std (X10)
2. U (Fig. 4; peak B)
3. U (Fig. 4; peak B)
4. POU (Fig.4; peak B)
5. N (Fig. 6; peak D)
6. U (Fig.6; peak D)
7. POU (Fig. 6; peak D)
8. HSA
9. Pig plasma
10. MW Std. (X10)

Plate 24

0.1% SDS - 4%to 20% gradient PAGE

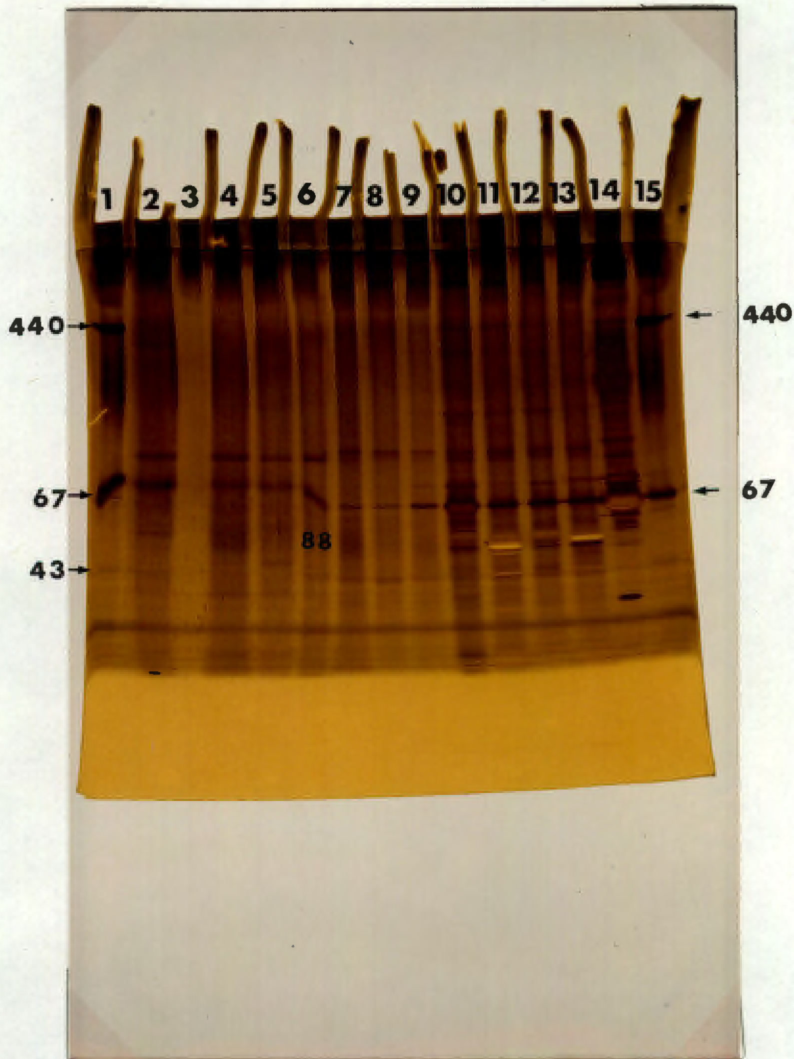


Slot No.

1. MW Std. ($X10^{-3}$)
2. 24 hr. C (Fig.4, peak A)
3. 48 hr. C (Fig. 4. peak A)
4. PU (Fig.4, peak A)
5. U (Fig. 4, peak A)
6. 48 hr.C (Fig.4,peak A) with MSH
7. PU (Fig. 4, peak A) with MSH
8. U (Fig. 4, peak A) with MSH
9. 24 hr C (Fig. 4, peak B)
10. 48 hr C (Fig. 4, peak B)
11. PU (Fig. 4, peak B)
12. U (Fig. 4, peak B)
13. POU (Fig.4, peak B)
14. Pig plasma
15. MW Std. ($X10^{-3}$)

Plate 25

0.1% SDS 4%to 20% gradient PAGE



Slot No.

1. MW Std. (X10^{-3})
2. U (Fig. 4, peak A) with MSH
3. ----
4. PU (Fig. 4, peak A) with MSH
5. 48 hr C (Fig.4, peak A) with MSH
6. U (Fig. 4, peak A)
7. PU (Fig.4, peak A)
8. 48 hr C (Fig.4, peak A)
9. 24 hr C (Fig.4, peak A)
10. U sm
11. PU sm
12. 48 hr. C sm
13. 24 hr.C sm
14. Pig plasma
15. MW Std. (X10^{-3})

Plate 26.

0,1% SDS-PAGE stained with BCB

Slot No.

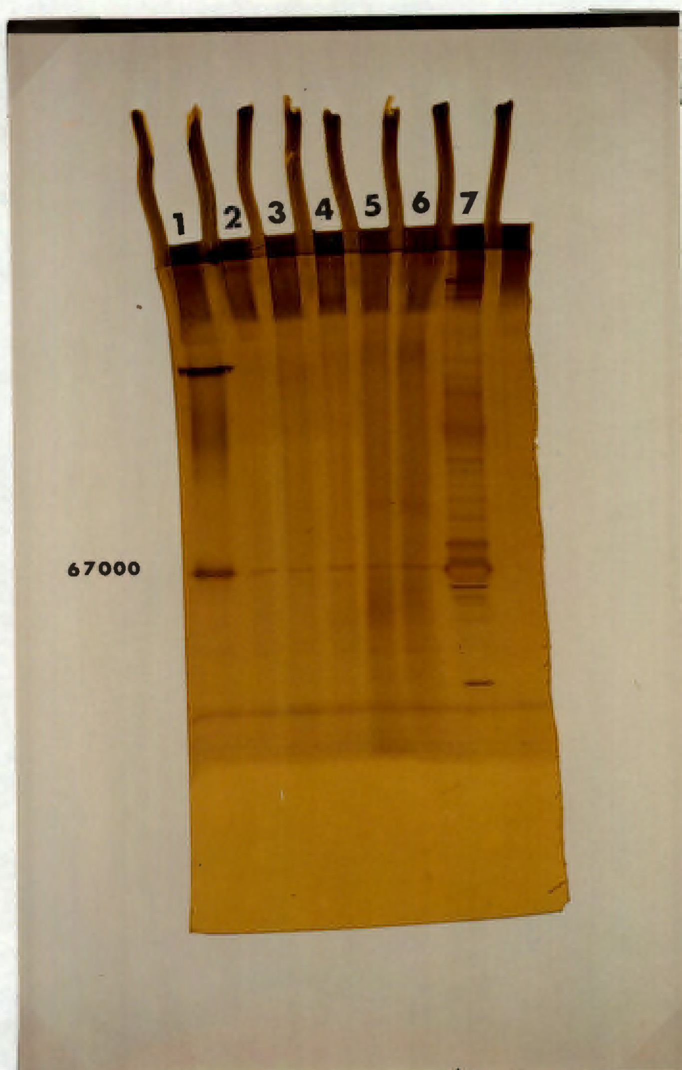
1. MW Std. 2. 24 hr.C (fig. 7) 3. POU (Fig. 7)

4. 24 hr.C (Fig.5, Fractions 5-10) 5. PU (Fig.5, Fractions 5-10)

6. POU (Fig.5, Fractions 5-10) 7. Pig plasma

Plate 27

0,1% SDS-PAGE stained with $AgNo_3$



Slot No.

1. MW Std.
2. 24 hr.C (Fig. 7)
3. POU (Fig.7)
4. 24 hr.C (Fig.5, Fractions 5-10)
5. PU (Fig.5, Fractions 5-10)
6. POU (Fig.5, Fractions 5-10)
7. Pig plasma

Plate 28

0.1% SDS - 4%to 20% gradient PAGE; Stained with NH_3AgNO_3

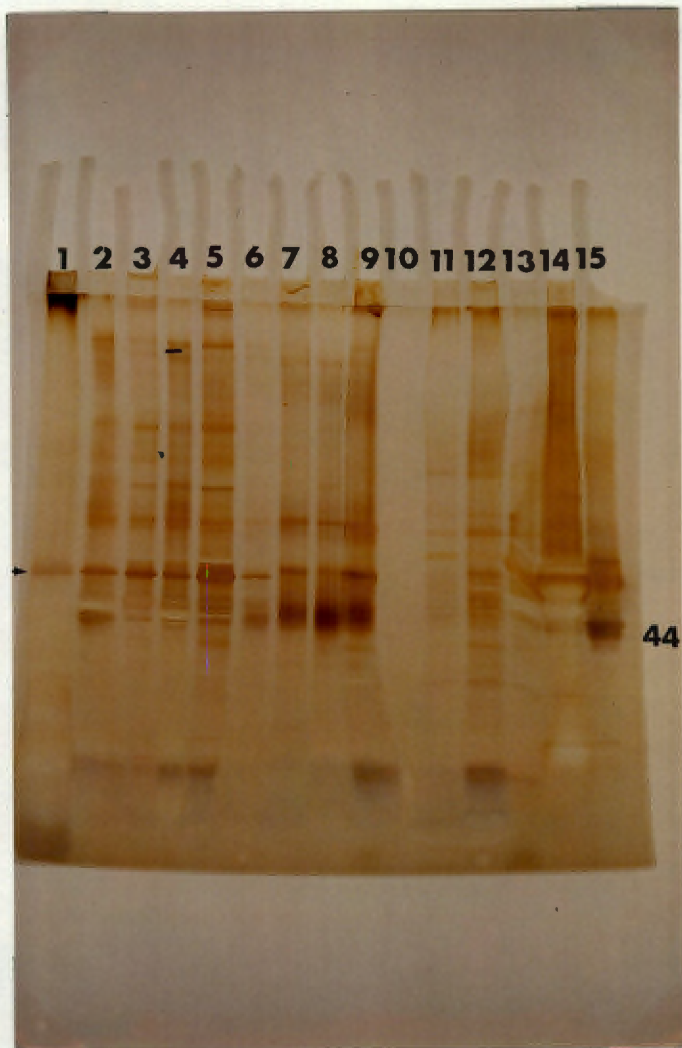
Slot No.

1. Pig gastric mucin (Sigma) 70,000 (arrow)
2. 24 hr.C (Fig. 7)
3. 48 hr.C (Fig. 7)
4. PU (Fig. 7)
5. U (Fig. 7)
6. POU (Fig. 7)
7. Ovine submaxillary glycoprotein (Sigma)
8. -
9. 24 hr.C (Fig. 7) with MSH
10. PU (Fig. 7) with MSH
11. U (Fig. 7) with MSH
12. 24 hr.C (Fig. 5, Fractions 5-9)
13. PU (Fig. 5, Fractions 5-9)
14. U (Fig. 5, Fractions 5-9)
15. POU (Fig. 5, Fractions 5-9)

Plate 29

0.1% SDS - 4%to 20% gradient PAGE stained with NH_3-$AgNO_3$

(Dubray et al.,1983)



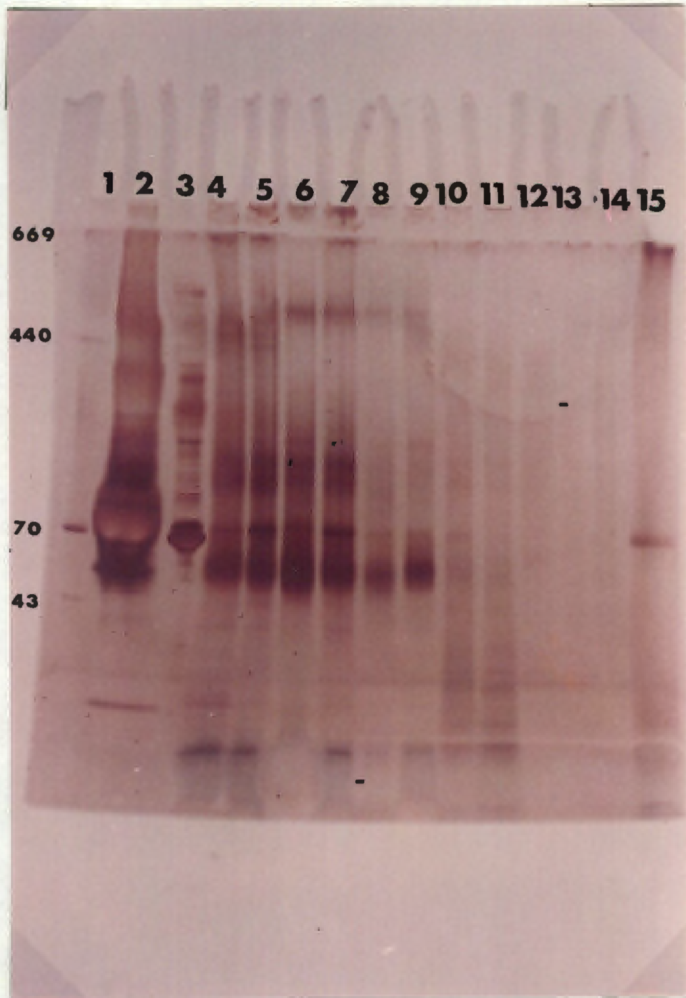
Slot No.

1. Pig gastric mucin (Sigma) M.W. 70,000 (arrow)
2. 24 hr. C sm
3. 48 hr. C sm
4. PU hr. sm
5. U sm
6. 24 hr C (Fig. 4, Peak A)
7. 48 hr C (Fig. 4, Peak A)
8. PU (Fig. 4, Peak A)
9. U (Fig. 4, Peak A)
10. -
11. 24 hr. C (Fig. 4, Peak A) with MSH
12. U (Fig. 4, Peak A) with MSH
13. 24 hr. C (Fig. 4, Peak B)
14. POU (Fig. 4, Peak B)
15. Ovine submaxillary glycoprotein (Sigma); M.W. ($x10^{-3}$)

Plate 30

0.1% SDS - 4%to 20% gradient PAGE; stained with NH_3-$AgNO_3$

(Dubray et al., 1983)



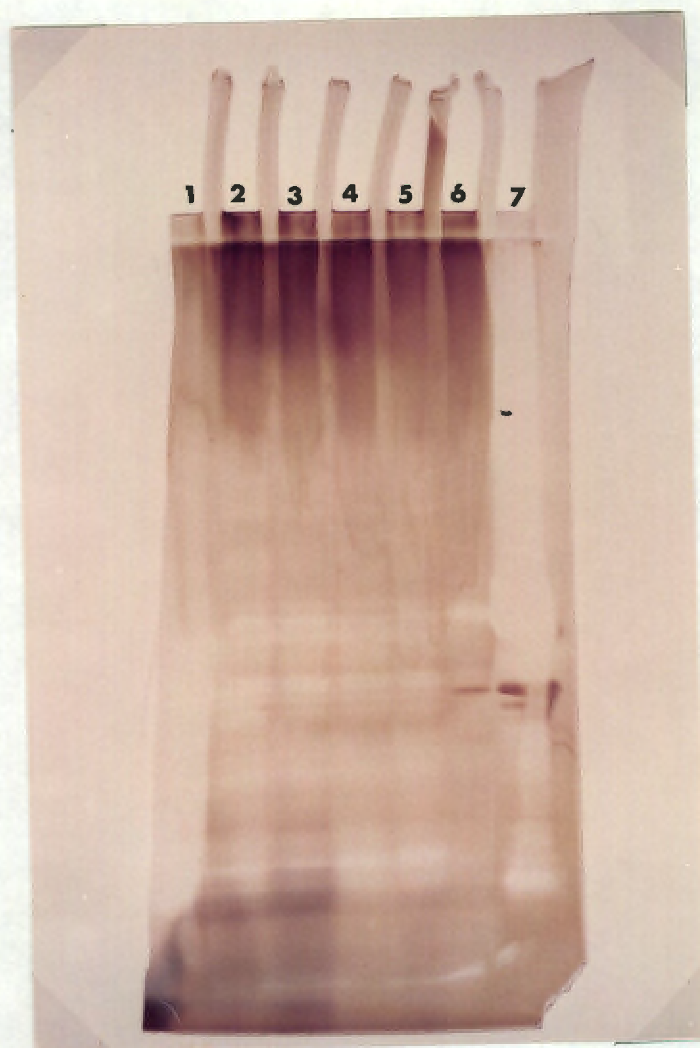
Slot No.

1. M.W. Standard
2. Ovine submaxillary glycoprotein
3. Pig plasma
4. POU (Fig. 5, Fractions 5-9)
5. U (Fig. 5, Fractions 5-9)
6. PU (Fig. 5, Fractions 5-9)
7. 48 hr. C (Fig. 5, Fractions 5-9)
8. 24 hr. C (Fig. 5, Fractions 5-9)
9. N (Fig. 5, Fractions 5-9)
10. POU (Fig. 7, Fractions 5-9)
11. U (Fig. 7, Fractions 5-9)
12. PU (Fig. 7, Fractions 5-9)
13. 24 hr. C (Fig. 7, Fractions 5-9)
14. N (Fig. 7, Fractions 5-9)
15. Pig gastric mucin (Sigma)

Plate 31

0.1% SDS - 4% to 20% gradient PAGE stained with NH_3-$AgNO_3$

(Dubray et al., 1983)



Slot No.

1. -
2. POU + MSH
3. U + MSH
4. PU + MSH
5. 48 hr. C + MSH
6. N (Fig. 7) + MSH
7. Ovine submaxillary glycoprotein.

4. CARBOHYDRATE ANALYSIS OF PORCINE GASTRIC MUCIN FROM NORMAL AND ULCERATED ANIMALS

4.1. Introduction

Analysis of the carbohydrate content of mucins requires the extensive purification of the glycoprotein themselves prior to the liberation and characterisation of the individual sugar constituents. Mucins are polydisperse in size, primarily because of the variation in the length of the oligosaccharide chains associated with the polypeptide core. Also the number of chains associated with each protein backbone varies considerably. Mucins show a large degree of heterogeneity in terms of side chains (Allen, 1981). Also there are considerable variations in the structure of the carbohydrate chains in glycoproteins from the stomach of different species (Allen,1981), and furthermore there are differences between glycoproteins from different anatomical areas in the same species. The size of the carbohydrate chains attached to the protein core varies from relatively simple disaccharide units to a complex oligosaccharide structure involving up to twenty-two sugars. Microheterogeneity between the side chains can occur as a result of biosynthetic variability or because of endogenous glycosidase activity in the gut.

The use of gas liquid chromatography for the analysis of sugar derivatives is well documented. The main advantages of gas-liquid chromatographic methods for the estimation of carbohydrate in mucins are their specificity and sensitivity (Oates et al., 1965). A combination of GLC with mass spectroscopy allows the unambiguous identification of the constituent sugars, appropriately derivatised.

GLC analysis of glycoproteins requires chemical derivatisation of the

monosaccharides obtained after hydrolysis of the parent glycoprotein. The application of this derivatisation procedure to the determination of the individual sugars constituting a polysaccharide involves several steps. First, the carbohydrate polymer must be treated with acid to release the component monosaccharides. Next, these nonvolatile monomers must be isolated from contaminating materials and then derivatised prior to GLC analysis. Spurious and interfering chromatographic peaks can result from both contamination of the original sample and contamination introduced by the reagents employed.

4.2 Materials and Methods

The blood groups of each of the animals was determined according to the method of Tovey et al., (1962). Mucins from normal, sham-operated and ulcerated pigs, purified as described earlier, were analysed for their component sugars by GLC. One milligram of PAS positive material was used for each analysis.

4.2.1 Hydrolysis and Derivatisation of Mucin Carbohydrate Chains.

Samples equivalent to a milligram of intact glycoprotein material were freeze-dried in hydrolysis glass ampoules. Each of the ampoules was then sealed after the addition of 500 ul of 2M TFA, and heated for 18 hours at 100°C. After hydrolysis the acid was removed on a rotary evaporator by co-distillation with methanol, and the sugars present in the hydrolysate were dissolved in water and reduced to alditols using sodium borohydride (Sloneker, 1972, Torello et al., 1980). Glacial acetic acid was used to destroy excess borohydride and the ensuing borate was removed as the volatile trimethyl borate by evaporation with methanol. This step is

essential since the borate formed from sodium borohydride is known to complex with the alditol derivatives, thereby interfering with their subsequent acetylation (Blakeney et al., 1983). Repeated evaporations with methanol/acetic acid were required to remove borates completely (Fox, 1983).

It is common practice to employ pyridine as a catalyst for the acetylation reactions. However, in our case its addition was unneccessary since sufficient sodium acetate, (also a catalyst) was generated during the methanol/acetic acid evaporations (Albertsheim, et al., 1967; Fox,1983). After the final evaporation to dryness using the methanol/acetic acid mixture, acetic anhydride (1,0 ml.) was added and samples were heated in a water bath for thirty minutes at 80^{0} C. Excess acetic anhydride was then removed by repeated evaporation with ethanol. Samples were diluted with water and extracted with chloroform. Chloroform extracts were dried over anhydrous sodium sulphate for 16 hours, filtered and then concentrated to dryness. Immediately prior to GLC analysis, the alditol acetate derivatives were dissolved in $CHCl_3$ for injection into the gas chromatograph.

4.2.2 Analysis of Alditol Acetates by GLC

The derived alditol acetates were examined by GLC using both packed and capillary columns.

Column (1) was a glass column, 6 m by 3mm I.D., packed with 3% OV-225 on 100-120 mesh Gas-Chrom Q and used in a Packard-Becker Gas Chromatograph, Model 433, fitted with a flame ionization detector. Nitrogen at a flow rate of 25 ml/min, was used as the carrier gas and routinely 0.5 ul samples

were injected. Samples were run isothermally at 230°C, with an injection temperature of 250°C and a detector temperature of 300°C. Standard alditol acetate derivatives of fucose, galactose and N-acetylglucosamine were injected under the same conditions (Fig. 13). Quantitations of individual sugars were performed using the integrator option which was part of the Model 433 chromatograph. The relative areas of the individual peaks were regarded as directly proportional to the molar ratios of the constituents of the sample and were expressed in relation to the area obtained for the galactose derivative which is arbitrarily assigned a value of unity.

Column (2) was a glass capillary column 25 m. x 0,35 mm. I.D. coated with OV-225 and used in a Carlo-Erba 4200 gas chromatograph, connected to a supergrator 3A computing integrator. Helium, at a flow rate of 75 $ml^{-1}.min^{-1}$ was used as a carrier gas and 1 ul samples were injected. Samples were run isothermally at 230°C for 4 minutes, followed by a 4°C/minute rise to 250°C this final temperature being maintained for 10 minutes.

Finally, to confirm the identification of the derivatized sugar; samples were further examined by GLC-mass spectrometry (see Appendix). GLC-MS was performed on a Carlo-Erba 4200 gas chromatograph coupled through a jet separator to a VG micromass 16F mass spectrometer. Helium was the carrier gas. A glass column, 2 m x 3 mm. I.D., packed with 3% OV-225 on chromosorb W-HP, 80-100 mesh, at 230°C (isothermal) was used to separate the derived alditol acetates.

4.3 Results and Discussion

From Table 6 it is evident that the fucose content of mucins decreased significantly in the ulcerated and post-ulcerated animals as compared with

the normal and control pigs (Figs. 14,15,16,17,18,19). The low levels of N-acetylgalactosamine found in samples from all the pigs was due to the fact that they were all O blood-group animals. A small decrease in N-acetylglucosamine content was observed with mucin from ulcerated and post-ulcerated as compared to normal animals but sham-operated animals showed a marked increase in N-acetylglucosamine (Table 6). Results obtained from both the packed and capillary columns were very similar.

These results appear to indicate these minor alterations in the proportion of constituent monosaccharides are indentifiable and occur concomitantly with ulceration of the stomach lining. This observation is consistent with a loss of the terminal, acid-labile fucose residues, possibly as a result of the decreased gastric pH.

It must be emphasized that the results of this study indicated only the relative proportions of the monosaccharide residues present in the mucins and not the absolute amounts of these constituents. In order to obtain information as to the specific structural changes which may be occurring in the oligosaccharide chains it would be necessary to remove these oligomers from the protein core and carry out an in depth investigation of their structures using standard carbohydrate techniques. While the changes in the proportions of galactose and fucose were quite apparent, there may also be smaller changes in the proportion of N-acetylhexosamine in these samples.

From the results obtained with the control (sham-operated) pigs there appeared to be a clear indication that the mucins isolated in these instances had a high proportion of N-acetylglucosamine (Table 6). This suggested that a different mechanism of secretion may be in operation under

(1978,1981) reported the occurrence of sulphate residues associated with the sixth carbon of N-acetylglucosamine at C-6. Histological studies at UCT Medical School have shown that the sulphated mucins identified in the cardiac gland area of pig stomachs were associated with cells of glandular origin, whereas surface mucins found in this region were non-sulphated. In the literature there have been suggestions that sulphated mucins are anti-ulcerogenic (Forstner et al. 1982) since they exhibit anti-pepsin activity. Drugs like amylopectin sulphate and sulphoglycopeptide have also become increasingly popular for treatment of ulcer patients since they too inhibit pepsin activity (Guslandi, 1980). It has also been suggested that the superficial mucus cells of the stomach secrete mucin as a consequence of direct mechanical or physical-chemical stimulation (from luminal contents), whereas secretion from deeper glands was brought about by neuronal mechanisms (Florey, 1955).

In the light of the above evidence we propose an increased secretion of glandular mucus in the cardiac gland area of our controls as a response to the traumatic stimuli of the sham operation. Further investigation regarding the sulphate content of pig gastric mucins from sham-operated pigs is clearly necessary.

Figure 14.

GLC profile of derivatised alditol acetates

1. Fuc; 2. Gal; 3. GalNAc; 4. GlcNAc

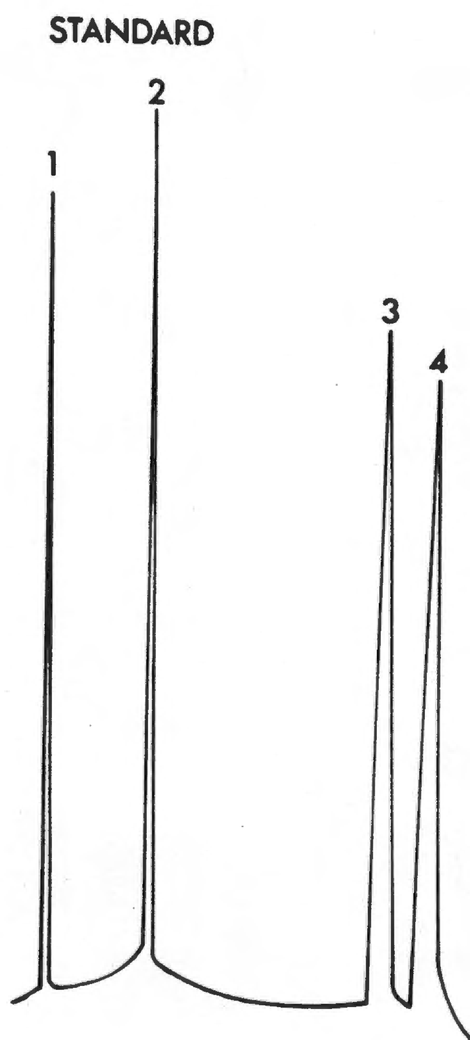


Figure 15

GLC profile of derived alditol acetates from a normal pig

1. Fuc; 2. Gal; 3. GalNAc; 4. GlcNAc

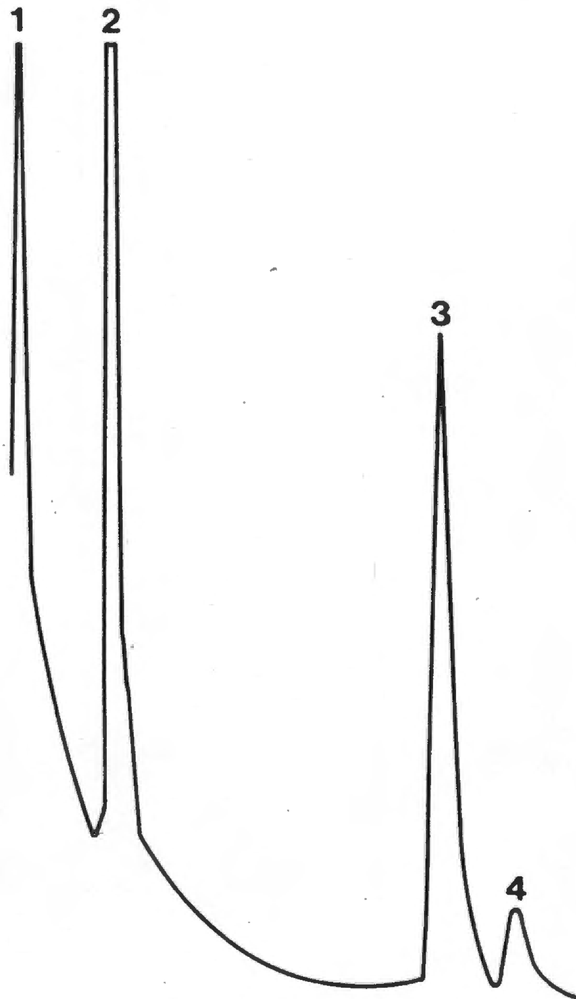


Figure 16

GLC profile of derived alditol acetates from a 24 hour control pig

1. Fuc; 2. Gal; 3. GalNAc; 4. GlcNAc

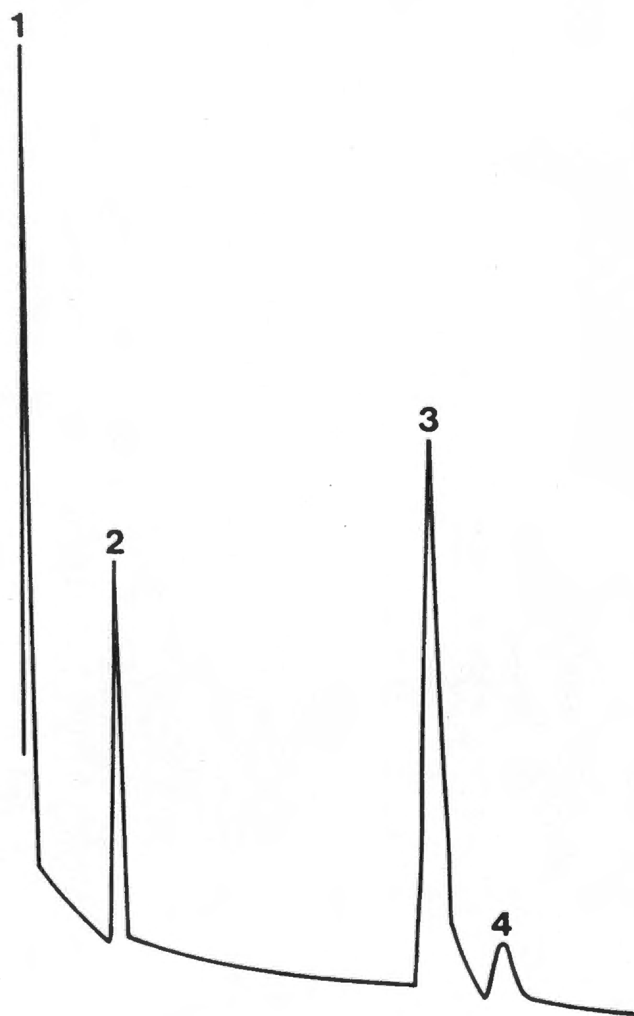


Figure 17

GLC profile of derived alditol acetates from a 48 hour control pig

1. Fuc; 2. Gal; 3. GalNAc; 4. GlcNAc

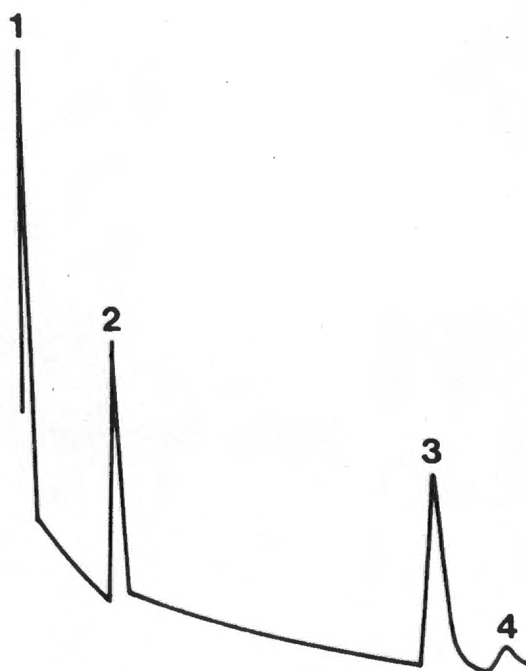


Figure 18

GLC profile of derived alditol acetates from a pre-ulcerated pig

1. Fuc; 2. Gal; 3. GalNAc; 4. GlcNAc

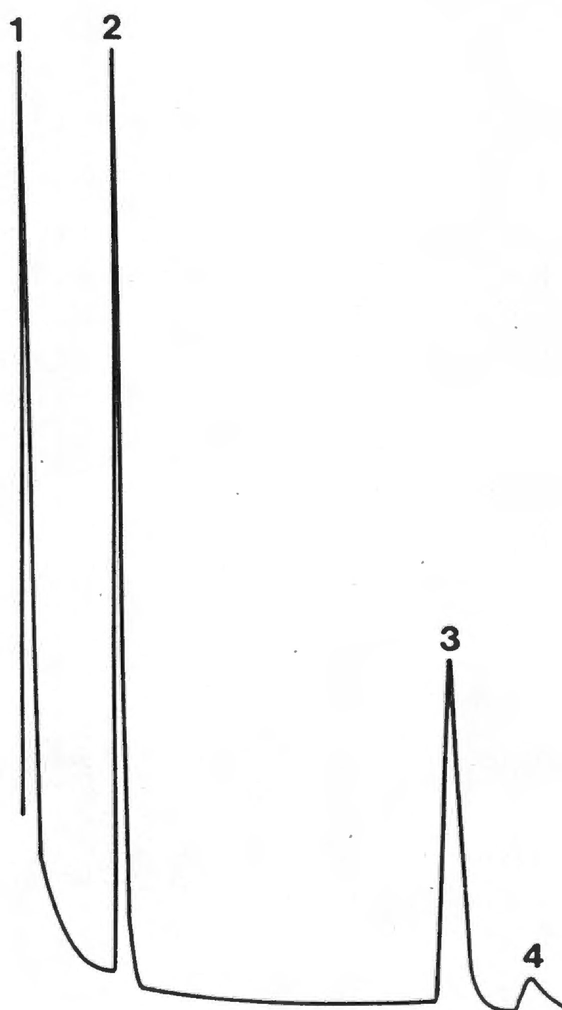


Figure 19

GLC profile of derived alditol acetates from an ulcerated pig

1. Fuc; 2. Gal; 3. GalNAc; 4. GlcNAc

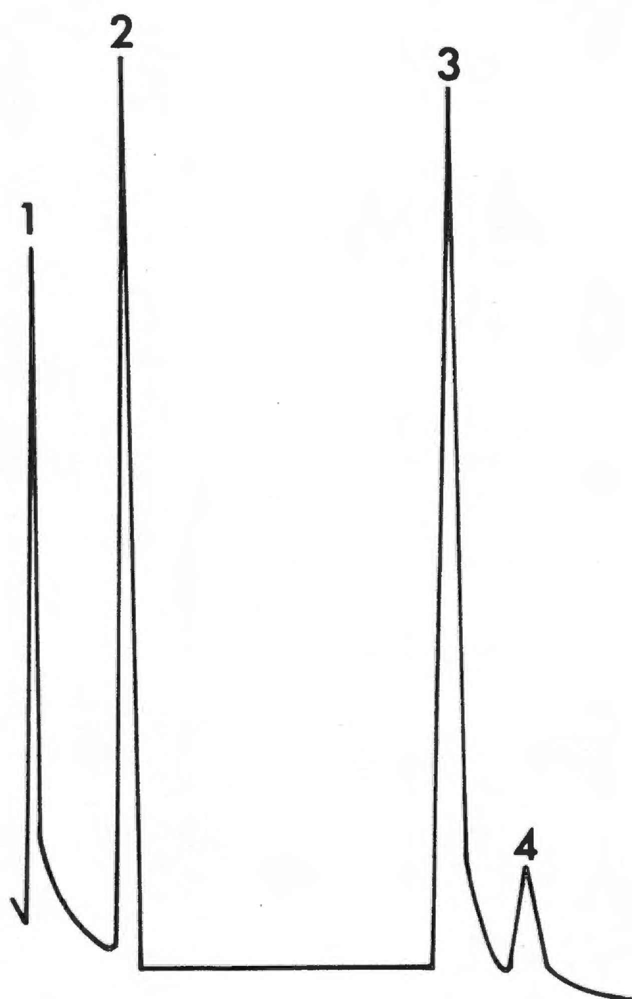


TABLE 6.

Ratios as determined by GLC of the constituent monosaccharides of pig gastric mucins, from the normal to the post-ulcerated states. (as determined on an OV-225 packed column)

	Fucose	Galactose	N-Acetyl-glucosamine	N-Acetyl-galactosamine
Normal	0,87 (0,7)	1,00 (1.00)	0,59 (0,59)	0,1 (0,08)
Sham-24 hours	1,00 (0,98)	1,00 (1.00)	4,52 (3,3)	0,55 (0,22)
Sham-48 hours	0,68 (0,60)	1,00 (1.00)	2.28 (1,59)	0,19 (0.14)
Pre-ulcerated	0,87 (0,78)	1,00 (1.00)	0,59 (0,619)	0,10 (0,118)
Ulcerated	0,47 (0,53)	1,00 (1.00)	0,72 (0,86)	0,32 (0,34)
Post-ulcerated	0,51 (0,68)	1,00 (1.00)	0,65 (0,61)	0,13 (0.08)

In parentheses: Capillary column results.

Note: Derivatives were prepared from aliquots taken from pooled fractions 2-5 after CsCℓ DGU (see Fig. 5).

5. CONCLUSION

Questions have arisen regarding the validity of the pig model in studies of the type presented in this thesis. Spontaneously developing ulcers have been reported to occur in various domesticated and laboratory animals (Kowalczyk, 1969). The pig is unique among experimental amimals in that peptic ulcers occur spontaneously in the pars oesophagea of the stomach, with a reported natural incidence of 25% or higher (Terblanche et al., 1978). This finding suggested that this animal might be useful for the laboratory study of peptic ulceration, including the study of methods of treatment. It was subsequently shown that bile duct ligation resulted in a 100% incidence of gastric ulceration of the squamous pars oesophagea of the pigs stomach (Arnot, 1974), with a 70% mortality rate from haemorrhage or perforation. It was also reported that the ulcers were similar, both in situation and histological appearance, to spontaneously occurring peptic ulcers (Terblanche, et al., 1978). These observations led to the use of the pig model in a composite study of gastric ulceration, which included the role of lysosomal enzymes (Berkman, 1979), the status of serum gastrin levels during ulceration (Castles et al., 1978), acid and pepsin levels (Hall, unpublished) and the present study of the role of mucus in normal and ulcerated states. This project became all the more important when it was reported (Pearson et al., 1980) that the pig and human gastric mucus were identical. It should be noted, however, that more recent studies have shown the structure of human gastric mucus to be somewhat more complex than previously reported (Clamp et al., 1983; Slomiany et al., 1983 and 1984 a). A further question has been raised about the experimental model in that the

actual site of ulceration, viz. the pars oesophagea, is specific to the pig and does not occur in humans. However, it cannot be disputed that the model has been useful in the establishment of biochemical techniques to study gastric mucins in general.

On the biochemical and clinical side the question that remains unanswered is whether the ulcerative process is a consequence of the degradation of mucus on the gastric mucosal surface. Further investigation by tissue culture of gastric mucosal epithelial cells may elucidate this point. In a preliminary study (Mall and Burden, unpublished work) we attempted to culture pig gastric mucosal epithelial cells, which were monitored for mucin synthesis for up to 24 hours. On application of the tissue culture medium to Sepharose CL-4B, the protein eluting at the void volume contained mucins of large molecular weight, an indication of <u>in vitro</u> synthesis. Bacterial contamination of the cells posed a major problem in this study necessitating the use of antibiotics in an amount that was five-fold greater than that used in studies reported in the literature (Yeomans et al., 1980). However, the tissue culture of pig or even human gastric mucosal cells seems feasible in the long term (Mohandas et al., 1982).

It has been reported that the pig gastric mucin molecule is heterogeneous (Allen, 1981), with the oligosaccharide side-chains ranging in length from 2-22 monosaccharide residues. It is to be expected therefore that the population of mucins in a single scrape of the pig gastric mucosa will vary accordingly. Greater variations can be expected in samples from mucosa in the ulcerated state.. Although, as reported in this thesis, changes in the monosaccharide content of pig gastric mucins

have been observed to accompany ulceration it is not possible at this stage to discern whether the observed changes are a result of an intracellular biosynthetic defect or whether they are due to extracellular carbohydrates, the activity of which may be greater in the ulcerated state. Therefore, in addition to the tissue culture of mucosal cells, isolation of the oligosaccharide chains of the mucins, in an intact form, seems imperative. This procedure has been applied in studies of O-glycosidically linked side-chain mucins from sources (Iyer et al., 1971; Slomiany et al., 1984 b) in which the carbohydrate side-chains were removed from the protein core by reduction with alkaline borohydride. Such an investigation is currently being pursued in this laboratory.

It has been mentioned that the large standard deviations obtained in assessments of the quantities of mucins present in mucosa necessitate the use of more sensitive techniques of measurement. Mantle et al., (1984) have reported the application of a radioimmunoassay technique in examination of mucins in rat small intestine. Dye-binding assays are still in use but in our experience reproducibility has proved to be a problem. It has been suggested that the measurement of the surface area could introduce a large error because of the folded surface of the stomach. The removal of the epithelial layer and its separation from the underlying mucosa first required the excision of the pars itself. Tearing of the epithelial layer during the process of separation was not an uncommon problem. It must be added that large standard deviations in biological experiments are frequently reported in the literature. The large standard deviations in the pre-ulcerated form cannot be explained.

In chapter 4 it was proposed that the high content of N-acetyl-

glucosamine in samples from the cannulated controls was a consequence of a defence mechanism of the pig's stomach, which secreted a higher concentration of sulphated mucins, the sulphates being linked to the C-6 of the GlcNAc residue (Allen, 1978). This proposal will have to be verified by the investigation of this sulphur content of mucins in cannulated pigs. In chapters 2 and 3 it was reported that the extent of proteolytic degradation of mucins from the 48 hr control samples was somewhat similar to that of samples from the ulcerated pig. Since we now have well established data on the times of the onset of the various sites of ulceration, viz. pre-ulcer, ulcer and post-ulcer, endoscopic monitoring of the development of the ulcer is no longer necessary and changes in mucus in normal and bile duct ligated pigs are now being studied without cannulae. Thus any variables caused by the traumatic effect of the cannula will be eradicated.

Terblanche et al., (1978) reported the prevention of peptic ulceration in the bile duct ligated pig by highly selective vagotomy. The gastric mucins of pigs that have undergone this operation are currently under study.

REFERENCES

Albersheim P, Nerins D.J., English P.D. and Karr A. Carbohydrate Research (1967) 5: 340.

Allen A., Pearson J.P. and Venables C.W. (1979) J.Physio. 293: 30p.

Allen A. and Garner A. (1980) Gut 21: 249-262.

Allen A. (1981) Biochem. Soc.Trans. Vol. 9, 2: 36p

Allen A. (1977) In: Mucus in Health and Disease. edited by Elstein M. and Parke Plenum Press. New York. 275-299.

Allen A. (1978) British Med.Bulletin. 34: 28-33.

Allen A., Pain R.H. and Robson T. (1976)

Allen A. and Snary D. (1972) Gut 13: 666-672

Allen A. and Starkey B.J. (1974). Biochim. et Biophys. Acta. 338: 364-368.

Allen A. (1981). In: Physiology of the Gastrointestinal Tract, edited by Leonard R.Johnson, Chapter 21: 617-639. Raven Press. New York.

Allen A., Bell A., Mantle M. and Pearson J. (1982) In: Mucus in Health and Disease II. edited by Chantler E.N., Elder J.B. and Elstein M. 144: 115-133. Plenum Press. New York.

Allen A. (1983) Trends in Biochemical Sciences 169-173.

Arnot R.S. (1974) ChM Thesis. University of Cape Town.

Bahari H.M.M., Ross I.N., and Turnberg L.A. (1982) Gut. 23: 513-516.

Berkman A.G. (1979) BSc.(Med)Hons. thesis, University of Cape Town.

Bickel M. and Kauffman G.L. (1981). Gastroenterology 80: 770-775.

Bhaskar K.R. and Reid L. (1981). J. Biol.Chem. 256: 14. 7583-7589.

Blakeney A.B., Harris P.J., Henry R.J. and Stone B.A. (1981) Carbohydrate Research 113: 291-299.

Boland C.R., Montgomery C.K. and Kim Y.S. (1982). Gastroenterology 82: 664-672.

Bolton J.P., Palmer D and Cohen M. (1978) Amer.J.Dig.Dis. 23: 4, 359-364.

Bonitati J., Elliott W.B. and Miles P.G. (1969). Analytical Biochemistry 31. 399-404.

Boursnell J.C., Hartree E.F. and Briggs P.A. (1970) Biochemical J. 117: 981-988

Bradford M.M. (1976). Analytical Biochemistry. 72: 248-254.

Brown D.T., Marriot C. and Beeson M.F. (1982). IN: Mucus in Health and Disease II edited by Chantler, R.N. Elder B.J. and Elstein M. 144: 85-88 Plenum Press. New York.

Carlson D.M. (1977) IN: Mucin in Health and Disease. Edited by Elstein M. and Parke D.V. 251-261. Plenum Press. New York.

Castles L.A., Terblanche J., van Hoorn-Hickman R. and Vinik A.I. (1978). Aust.NZ.J.Surg. 48: 2, 214-219.

Chantler E. (1982) IN: Mucus in Health and Disease. Edited by Chantler E.N., Elder J.B. and Elstein M. 144: 252-263. Plenum Press. New York.

Clamp J.R., Bhatti T. and Chambers R.E. (1972). In: Glycoproteins, Their Composition, Structure and Function. Part B. edited by A.Gottschalk. Elsevier, Amsterdam.

Clamp J.R. (1977) In: Mucus in Health and Disease. edited by Elstein M and Parke D.V. 1-14. Plenum Press. New York.

Clamp J.R. (1978) Brit. Med. Bull. 34: 1. 25-27.

Clamp J.R. (1980) In: Recent Advances in Gastrointestinal Pathology. Edited by Wright K. 47-58. W.B.Saunders Company. Ltd.

Clamp J.R., Cooper B., Creeth J.M., Ene D., Barret J. and Gough M. (1983). Biochem J. 215: 421-423.

Creeth J.M. and Denborough M.A. (1970) Biochem.J. 117: 879-891.

Creeth J.M. (1978) Br.Med.Bull. 34: 1. 17-24.

Davenport H.W. (1964) Gastroenterology 46: 3 245-253.

Davenport H.W., Warner H.A. and Code C.F. (1964) Gastroenterology 57: 142-152.

Davenport H.W. (1965) Gut. 6: 513.

Davenport H.W. (1967) The New Eng. J. of Med. 276: 1307-1312.

Davenport H.W. (1972) Scientific American 226: 87-93.

Dekanski J.B., MacDonald A, Sacra P. and Parke D.V. Brit. J. Pharm. (1975) 55: 387-392.

Domschke W., Domschke S., Classen M. and Demling L. (1972) Gastroenterology 7: 647-651.

Domschke S., Domschke W. (1984) Clinics in Gastroenterology 13, 405-436.

Dubray G. and Bezard G. (1982) Analytical Biochemistry. 119: 325-329.

Edwards P.A. (1978). Brit. Med. Bull. 34: 1. 55-56.

Ehsanullah M., Filipe M.I. and Gazzard B. (1982) Gut 23: 485-489.

Elder, J.B. and Hearn A.R. (1982) IN: Mucus in Health and Disease II Edited by Chantler R.N., Elder B.J. and Felstein M. 144: 197-198. Plenum Press. New York.

Elstein M and Parke D.V. (1977) In: Mucus in Health and Disease. xi-xii. Plenum Press. New York.

Elstein M. (1978) Br. Med. Bull. 34: 1. 83-88.

Elstein M. (1982) IN: Mucus in Health and Disease II Edited by Chantler R.N., Elder B.J. and Elstein M. 144: 301-318. Plenum Press New York.

Eschenbruch M. and Burk R.R. (1982) Analytical Biochemistry 125: 96-99

Fairbanks G., Steck T.L. and Wallach D.F.H. (1971) Biochemistry. 10: 13. 2606-2617

Fenster L.F. (1973) Medical Clinics of North America. 57: 5. 1289-1294.

Flemstrom G. (1981) IN: Physiology of the Gastrointestinal Tract. Edited by Johnson L.R. 603-616. Raven Press. New York.

Florey H. (1955). Proceedings of the Royal Society of London. 143: 147-158.

Florey H.W. (1962) Gastroenterology 43: 326-329.

Flynn A.M. and Lynch S.S. (1976) Br.J.Obstets and Gyn. 83: 656-659.

Fogelson S.J. (1931). J. Am. Med. Ass. 96: 9. 673-675.

Forstner J.F., Jabbal I. and Forstner G. (1973) Canadian J.Biochem. 51: 1154-1166.

Forstner I.F., and Forstner G.G. (1975) Biochimica et Biophysica Acta. 386: 283-292.

Forstner J.F. (1978). Digestion 17: 234-263.

Forstner G., Wesley A. and Forstner J. (1982). IN: Mucus in Health and Disease II. Edited by Chantler K.N., Elder B.J. and Elstein M. 144: 199-229 Plenum Press. New York.

Fox A. (1983) J. of Chromatography. 256: 429-438.

Frates R.C., Kaizu T.K., and Last J.A. (1983). Pediatr.Res. 17: 30-34.

Fujuta T. and Kobayashi S. (1981). IN: Gut Hormones 2nd edition. Edited by Bloom S.R. and Polak. J.M. 90-95. Churchill Livingstone publishers.

Gallagher J.T. and Richardson P.S. IN: Mucus in Health and Disease II. Edited by Chantler E.N., Elder J.B. and Elstein M. 144: 335-350. Plenum Press. New York.

Gaafar S.M. and Keittevuti B. (1972). Gastroenterology. 63: 423-426

Garner A., Flemstrom G. and Heylings J.R. (1979). Gastroenterology. 451-457

Gelman R.A. (1977) IN: Mucus in Health and Disease. Edited by Elstein M. and Parke D.V. 171-180. Plenum Press. New York.

Gibbons R.A. (1972). IN: Glycoproteins (2nd edition) Part A. Edited by A.Gottschalk. 31-109. Elsevier. Amsterdam.

Gibbons R.A. (1978) Br.Med.Bull. 34: 1. 34-38.

Gibbons R.J. and Qureshi J.V. (1978) Infection and Immunity 22: 3. 665-671

Glass G.B.J., Ishimori A. and Buckwalter J.A. (1962). Gastroenterology. 42: 443-454.

Glass G.B.J. and Slomiany B.L. (1977) In: Mucus in Health and Disease edited by Elstein M. and Parke D.V. 311-347. Plenum Press. New York.

Green A.P., Lander J.E. and Turner D.H. (1981). J.Pharm Pharmacol 33: 348-352.

Grossman M.I. (1979) Scand. J. Gastroenterology 15: 58. 7-16.

Guslandi M. (1978). Lancet i. 1267.

Guslandi M. (1980) Int.J. of Clinical Pharmacology, Therapy and Toxicology. 18: 3: 140-143.

Guth P.H. (1982). 33: 183-196.

Hafez E.S.E. (1977) IN: Mucus in Health and Disease Edited by Elstein M. and Parke D.V. 19-35. Plenum Press. New York.

Hakkinen I., Jarvi O. and Gronroos J. (1968) Intern.J. of Cancer. 3: 582-592.

Hall R.L., Miller R.J., Peatfield A.C. Richardson P.S., Williams I. and Lampert I. (1980) Biochem. Soc. Trans. 8: 1. 72.

Hawtrey A.O.,Scott-Burden T. and Robertson G. (1974). Nature, 252: 5478, 58-60.

Heatley N.G. (1959). Gastroenterology 37: 313-318.

Helander H.F. (1981) IN: Intern. Review of Cytology. 70: 217-289. Academic Press Incorporated.

Henry R.J., Blakeney A.B., Harris P.J. and Stone B.A. (1983) J. of Chromatography 256: 419-427.

Hill H.D.Jnr. Schwyzer H., Steinman H. and Hill R.L. J.of Biol.Chem 252: 3799-3804.

Holden K.G., Yim N.C.F., Griggs L.J. and Weisbach J.A. (1971) Biochemistry 10:16. 3110-3113.

Hollander F. (1954) Arch. Internal Med. 93: 107-120.

Hollander F. (1961). Gastroenterology 40: 4. 477-490.

Hoskins L.C. (1978). IN: The Glycoconjugates II. Edited by M.I.Horowitz and W.Pigman. 235-250. Academic Press. New York.

Isenberg J.I. (1975). Post Graduate Med. 57: 1. 163-168.

Ito S. (1981). IN: Physiology of the Gastrointestinal Tract. Edited by Leonard R. Johnson. 517-550. Raven Press. New York.

Ivy A.C., Grossman M.I. and Bachrach. W.H. (1950). IN: Peptic Ulcer. Churchill Publications. London.

Iyer R.N. and Carlson D.M. (1971). Arch. of Biochem. and Biophys. 142. 101-102.

Johansson C. and Bergstrom S. (1982). Scand. J. of Gastroen. 77: 21-46.

Johansson C. and Kollberg B. (1980) Scand. J. of Gastroen. 15: 93-97.

Johnston B., Symons A.M. and Parke D.V. (1975) Biochem Soc. Trans. 3: 1112-1115.

Jones R. and Reid L. (1978). Brit. Med. Bull. 34: 1. 9-16.

Jones Sir F.Avery- (1977) IN: Mucus in Health and Disease. Edited by Elstein M. and Parke D.V. Plenum Press. New York. vii-ix.

Jones Sir F.Avery- (1978) Br.Med.Bull. 34: 1. 1-4.

Kagnoff M.F., Donaldson R.M., Trier J.S. (1972). Gastroent. 63: 541-551.

Karsner H.T. (1942) IN: Human Pathology, 6th Ed., J.P.Lippincott Co., Philadelphia.

Kashket S. and Donaldson C.G. (1972). J. of Bacteriol. 112: 3. 1127-1133.

Kim Y.S., Isaacs R. and Perdomo J. (1974). Proc. of the National Acad. of Sci. 71: 4869-4873.

Kivilaakso E., Silen W. (1979) New Eng.J.Med. 301: 7. 364-369.

Klaas J. II (1974) J. of Parasitology. 60: 6. 907-910.

Koffman C.G., Berry J and Elder J.B. (1977) Brit. J. of Surg. (Abstract) 64: 830.

Kowalczyk T. (1969) Am.J.Vet.Res. 30: 3, 393-399.

Lamont J.T. and Ventola A. (1977). Gastroent. 72: 82-86.

Lamont J.T. and Ventola A.S. (1980) Biochimica et Biophysica Acta. 626: 234-243.

Iyer R.N. and Carlson D.M. (1971). Arch. of Biochem. and Biophys. 142. 101-102.

Johansson C. and Bergstrom S. (1982). Scand. J. of Gastroen. 77: 21-46.

Johansson C. and Kollberg B. (1980) Scand. J. of Gastroen. 15: 93-97.

Johnston B., Symons A.M. and Parke D.V. (1975) Biochem Soc. Trans. 3: 1112-1115.

Jones R. and Reid L. (1978). Brit. Med. Bull. 34: 1. 9-16.

Jones Sir F.Avery- (1977) IN: Mucus in Health and Disease. Edited by Elstein M. and Parke D.V. Plenum Press. New York. vii-ix.

Jones Sir F.Avery- (1978) Br.Med.Bull. 34: 1. 1-4.

Kagnoff M.F., Donaldson R.M., Trier J.S. (1972). Gastroent. 63: 541-551.

Karsner H.T. (1942) IN: Human Pathology, 6th Ed., J.P.Lippincott Co., Philadelphia.

Kashket S. and Donaldson C.G. (1972). J. of Bacteriol. 112: 3. 1127-1133.

Kim Y.S., Isaacs R. and Perdomo J. (1974). Proc. of the National Acad. of Sci. 71: 4869-4873.

Kivilaakso E., Silen W. (1979) New Eng.J.Med. 301: 7. 364-369.

Klaas J. II (1974) J. of Parasitology. 60: 6. 907-910.

Koffman C.G., Berry J and Elder J.B. (1977) Brit. J. of Surg. (Abstract) 64: 830.

Kowalczyk T. (1969) Am.J.Vet.Res. 30: 3, 393-399.

Lamont J.T. and Ventola A. (1977). Gastroent. 72: 82-86.

Lamont J.T. and Ventola A.S. (1980) Biochimica et Biophysica Acta. 626: 234-243.

Lamont J.T. (1981) IN: Basic Mechanisms of Gastrointestinal Mucosal Cell Injury and Protection. Edited by John W.Harmon. 391-402. Williams & Wilkins.

Lamont J.T., Ventola A.S., Maull E.A., Szabo S. (1983). Gastroent. 84: 2. 306-313.

Levine M.J., Herzberg M.C., Levine M.S., Ellison S.A., Stinson M.W., Li H.C. and van Dyke T. (1978) Infection and Immunity. 19: 1. 107-115.

Lewin M.R., El Masssri S.H. and Clark C.G. (1979) Eur. Surg.Res. 11: 392-398.

Lopez-Vidriero M.T. and Reid L. (1978). Br.Med.Bull. 34: 1. 63-74.

Lowry O.H., Rosebrough N.J., Farr A.L. and Randal K. (1951). J.of Biol.Chem 193: 265-275.

Mantle M. and Allen A. (1978) Biochem.Soc. Transactions. 6: 20: 607-609

Mantle M., Mantle D. and Allen A. (1981). Biochem. J. 195: 277-285.

Mantle M. and Allen A. (1981). Biochem.J. 195: 267-275.

Mantle M., Forstner G. and Forstner J.F. (1984). J. of Biol.Chem. 217. 159-167.

Marriott C. (1982). IN: Mucus in Health and Disease' Edited by Chantler E.N., Elder J.B. and Elstein M. 144: 75-84. Plenum Press. New York.

Marshall T. and Allen A. (1977). Biochemical Society Transactions. 5: 436-439.

Martin G.P., Marriott C. and Kellaway I,W. (1978) Gut 19: 103-107.

Menguy R. and Masters Y.F. (1965). Surg. Gynaec. and Obsts. 120: 92-98.

Menguy R. (1969). Amer.J. Surg. 117: 806-812.

Meyer F.A., Vered J. and Sharon N. (1977). IN: Mucus in Health and Disease I. Edited by Elstein M. and Parke D.V. 239-249. Plenum Press. New York

Mohandas T.K., Lechago J. and Rotter J.I. (1982).(abstract) gastro-Gastroenterology 82: 5, Part 2. 1133.

Moody F.G. and Zalewsky C.A. (1981). IN: Basic Mechanisms of Gastro-intestinal Mucosal Cell Injury and Protection. Edited by John W.Harmon. 373-389. Williams and Wilkins Publishers.

Morris E.R. and Rees D.A. (1978). Brit. Med. Bull. 34: 1. 49-53.

Negus V. (1967). Proceedings of Royal Society of Medicine. 60: 75-84.

Neutra M. and Leblond C.P. (1966). J. of Cell Biology. 30: 137-150.

Nilson B., de Luca S., Lohmander s. and Hascall V.C. (1982). J. of Biol. Chem. 257. 18, 10920-10927.

Nirmala V. and Thomas J.A. (1982). IN: Mucus in Health and Disease II. Edited by Chantler R.N., Elder B.J. and Elstein M. 285-287. Plenum Press, New York.

Oakley B.R., Kirsch D.R. and Morris N.R. (1980) Analytical Biochemistry 105: 361-363.

Oates M.D.G. and Schrager J. (1965). Biochem. J. 97: 697.

Parke D.V. (1976). "Topics in Gastroenterology" 4: 329-343. edited by Truelove S.C. and Ritchie J.A. Blackwell Scientific Publications.

Parke D.V., and Symons A.M. (1977). In: Mucus in Health and Disease edited by Elstein M. and Parke d.V. 311-347. Plenum Press. New York.

Parke D.V. (1978) Brt. Med.Bull. 34: 1. 89-94.

Pearson J., Allen A. and Venables C.W. (1979) Biochem. Society Tran. 7:5. 904-905.

Pearson J., Allen A. and Venables C. (1980) Gastroenterology 78: 709-715.

Pearson J. and Allen A. (1980) Biochem. Soc. Trans. 8: 3. 388-389.

Pearson J.P., Allen A. and Parry S. (1981) Biochem. J. 197: 155-163.

Pearson J.P. and Allen A. (1982). IN: Mucus in Health and Disease II Edited by Chantler R.N., Elder B.J. and Elstein M. 144: 151-153. Plenum Press, New York.

Phelps C.F. (1978) Br.Med.Bull. 34: 1. 43-48.

Pringle R. (1977). IN: Mucus in Health and Disease. Edited by Elstein M. and Parke D.V. 227-237. Plenum Press. New York.

Prout W. (1824) IN: Philosophical Transactions of the Royal Society, 1, 45-49.

Rees W.D.W and Turnberg L.A. (1980). Lancet ii. 410-412.

Rees W.D.W. and Turnberg L.A. (1981). Clinics in Gastroenterology. 10: 3, 521-546.

Rees W.D.W. and Turnberg L.A. (1982). Clinical Science 62: 343-348.

Reid L. and Clamp J.R. (1978). Br.Med.Bull. 34: 1, 5-8.

Reid L., Bhaskar K. and Coles S. IN: Mucus in Health and Disease II. Edited by Chantler E.N., Elder J.B. and Elstein M. (1982). 144: 369-391. Plenum Press, New York.

Roberts, N.B. and Taylor W.H. (1973). Clinical Science. 45: 213-224.

Robson T., Allen A. and Pain R.H. (1975) Biochem. Soc. Trans. 3: 1105-1109.

Ross I.N., Habib M.M. and Turnberg L.A. (1981) Gastroenterology 81: 713-718.

Sammons D.W., Adams L.D. and Nishizawa E.E. (1981). Electrophoresis 2: 135-141.

Scawen M and Allen A. (1977) Biochem. J. 163: 363-368.

Schachter H., McGuire E.J. and Roseman S. (1971). J. of Biol. Chem. 246: 5321-5328.

Schachter H. and Williams D. (1982). IN: Mucus in Health and Disease II. Edited by Chantler R.N., Elder B.J. and Elstein M. 144: 3-28. Plenum Press. New York.

Schrager J. and Oates M.D.G. (1968) Biochem. J. 106: 523.

Schrager J. and Oates M.D.G. (1970) Digestion 3: 231-242.

Schrager J. and Oates M.D.G. (1973). Gut 14: 324-329.

Schrager J. and Oates M.D.G. (1974). Biochim and Biophys.Acta. 372: 183-195

Schrager J and Oates M.D.G. (1978) Brit. Med Bulletin. 34: 1. 79-82

Segal I. (1983). Med.Chronicle 13: Conlyn House Publications. Johannesburg.

Seifter S., Dayton s., Novic B. and Muntwyler L.J. (1950). Arch. Biochem. & Biophys. 25: 191-200.

Shillingford J.S., Lindup W.E. and Parke D.V. (1974). Biochem.Soc. Trans. 2:1104-1107.

Silberberg A. and Meyer F.A. (1982). IN: Mucus in Health and Disease II. Edited by Chantler E.N, Elder J.B. and Elstein M. 144: 53-74. Plenum Press. New York.

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

Slomiany A., Witas Henryk, Mitsuru A. and Slomiany B.L. (1983). J.Biol.Chem 258: 14. 8535-8538.

Slomiany A., Jozwiak Z., Takagi A. and Slomiany B.L. (1984 a). Arch. of Biochem and Biophys. 229. 2, 560-567.

Slomiany B.L., Zdebska E., and Slomiany A. (1984 b). J. of Biol. Chem. 259. 5. 2863-2869.

Sloneker J.H. In. Methods in Carbohydrate Chemistry. (1972) Vol. 6. (Whistler R.L. and B.E.Miller). J. N.Eur. Academic Press. New York 20.

Sloss M.W. (1932). MSc. Thesis.

Sloss M.W. (1954). Amer. J. Vet.Res. 578-593.

Smith B.F. and Lamont J.T. (1983). Gastroenterology. 85: 707-712.

Snary D. and Allen A. (1971). Biochem. J. 123: 845-853.

Snary D., Allen A. and Pain R.H. (1972). (Abstract) Biochem. J. 127: 68P-69P.

Snary D., Allen A. and Pain R.H. (1972). (Abstract) Biochem.J. 128: 123P-124P.

Snary D., Allen A. and Pain R.H. (1974). Biochem.J. 141: 641-646.

Solcia E., Capella C., Buffa R., Usellini L., Fiocca R. and Sessa F. (1981). IN: Physiology of the Gastrointestinal Tract. Edited by Leonard R.Johnson . 39- 58. Raven Press. New York.

Starkey B.J., Snary D. and Allen A. (1972) (Abstract). Biochem.J. 128: 123P.

Starkey B.J., Snary D. and Allen A. (1974) Biochem. J. 141: 633-639.

Strombeck D.R. and Harrolf D. (1974). Infection and Immunity. 10: 6, 1266-1272.

Subbuswamy S.G. (1971). Gut. 12: 200-207.

Switzer R.C., Merril C.R. and shifrin S. (1979) Analytical Biochem. 98: 231-237.

Syner F.N. and Moghissi K.S. (1972) Biochemical J. 126. 1135-1140.

Teorell T. (1939) J. Gen.Physiol. 23: 263-274.

Teorell T. (1940) J.Physiol. 97: 308-315.

Terblanche J., van Hoorn-Hickman R. (1978). Surgery 84: 206-211.

Thjodleifsson B. and Wormsley K.G. (1977). Digestion. 15: 53-72.

Tomasi T.B. (1972). N.Eng. J. Med. 287: 10. 500-506.

Torello L.A., Yates, A.J. and Thompson D.K. (1980). J. Chromatography. 202. 195-209

Vagne M. and Perret G. (1976). Scand. J. Gastroenterology. 42: 63-73.

Van Huis G.A. and Kramer M.F. (1981) Gut 22: 782-787.

Van Kooij R.J., Roelofs H.J.M., Kathmann G.A.M. and Kramer M.F. (1980). Fertility and Sterility. 34: 3. 226-233.

Van Kooij R.J. and Kramer M.F. (1982) IN: Mucus in Health and Disease II. edited by Chantler R.N., elder B.J. and Elstein M. 144: 271-272. Plenum Press, New York.

Watkins W,M. (1966). Science. 152: 172-181.

Watson R.G., Vinik A.I., van Hoorn-Hickman R. and Terblanche J. (1978). S.Afr.J.Med. 54: 1019-1021.

Wesley A., Forstner J., Quereshi R., Mantle M and Forstner G. (1983). Pediatr. Res. 17: 65-69.

Wesley A.W., Forstner J.F. and Forstner G.G. (1983). Carbohydrate Res. 115: 151-163.

Williams S.E. and Turnberg L.A. (1979). (Abstract) Gut. 20: A922-A923.

Williams S.E. and Turnberg L.A. (1980). Gastroent. 70: 299-304.

Williams S.E. and Turnberg L.A. (1981). Gut. 22: 94-96.

Yeates D.B. (1982). IN: Mucus in Health and Disease II. edited by Chantler E.N., Elder J.B. and Elstein M. 144: 411-415. Plenum Press. New York.

Yeomans N.D. and Millar S.J. (1980). Dig. Dis. and Sci. 25. 4, 295-301.

Younan F., Pearson J. Allen A. and Venables C. (1982). Gastroenterology 82: 827-831.

Zalewsky C.A. and Moody F.G. (1979). Gastroent. 77: 719-729.

Appendix A

Scrape of mucus

|

homogenization for 30 seconds in CH_3COO^-/CH_3COOH buffer pH 5,8, 0.2 M NaCl 0.02% Na azide.

|

spin 23000 g for 10 minutes

|

supernatant — pellet discarded

Sepharose CL-4B

peak A — peaks B and C (Fig. 4)

peaks B and C → Sephacryl S-300 (Fig. 6)

|

Isopycnic centrifugation in CsCl 105000 g for 10 minutes (Fig. 5)

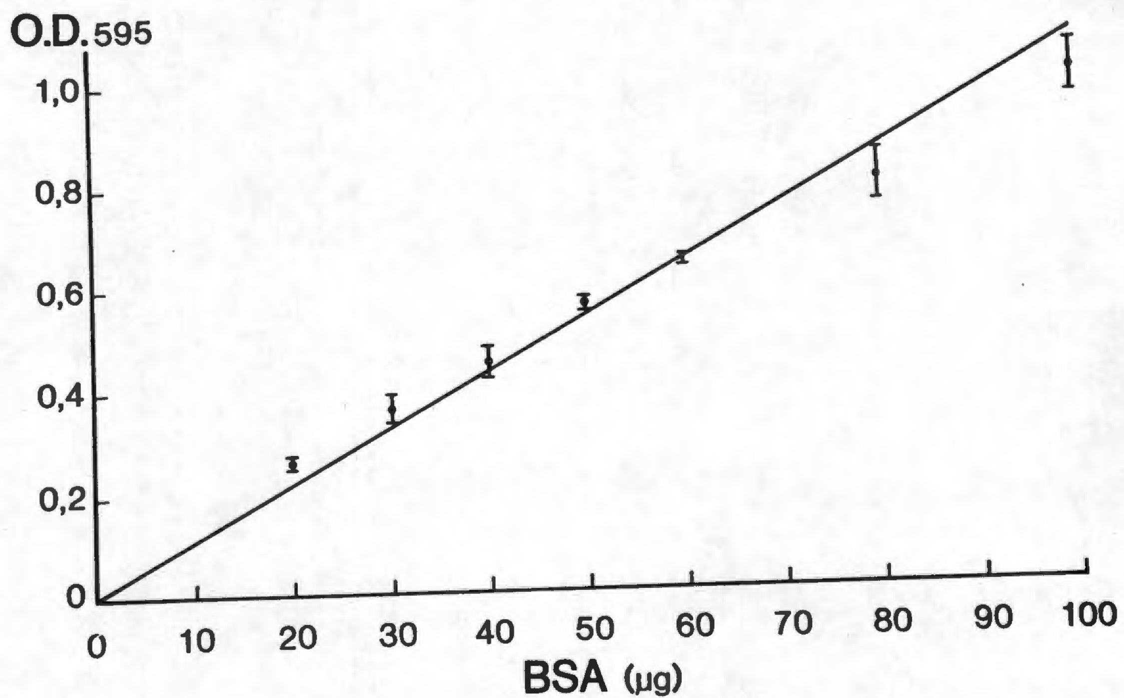
|

carbohydrate rich fractions of high density (pure mucins) — contaminant protein fractions of low density

|

Sepharose CL-2B (Fig.7), pure mucins

The Bio-Rad Assay



ug.	Absorbance 595 nm	
10	0.139 ± 0.0007	n=5
20	0.256 ± 0.018	"
30	0.358 ± 0.033	"
40	0.451 ± 0.028	"
50	0.560 ± 0.018	"
60	0.638 ± 0.011	"
80	0.809 ± 0.055	"
100	1.0 ± 0.046	"

Appendix B

The Bio-Rad assay is a highly reproducible dye-binding assay with almost no interference from carbohydrates in large quantities (Bradford, 1976), sodium chloride and 0,1% sodium dodecyl sulfate.

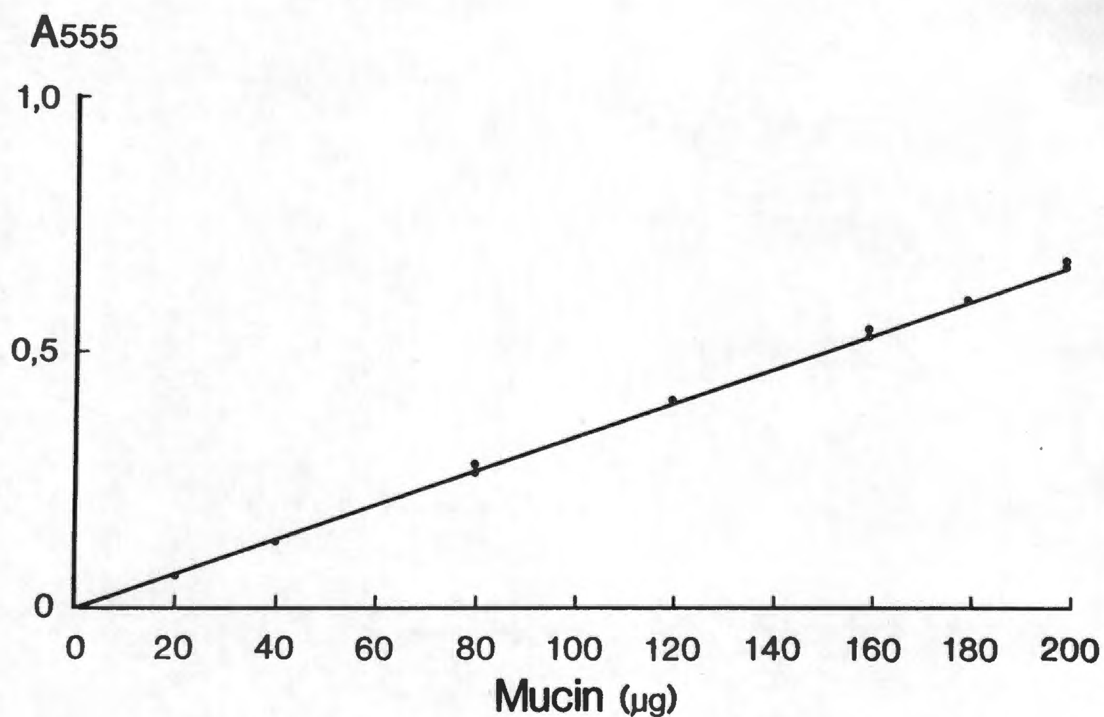
These factors made the Bio-Rad reagent suitable for the assay of mucus glycoproteins of carbohydrate levels of 70% and a high cysteine content in a buffer system containing 0.2M NaCl.

The Bio-Rad Standard Curve

Bovine serum albumin, fraction V, at a concentration of 1 mg/ml was prepared.

The standard curve was initially done five times, to determine its reproducibility.

Absorbances obtained at A_{555} for various concentrations of mucin



ug.(mucin)	A	A
20	0.059	0.059
40	0.124	0.128
60	0.192	0.192
80	0.260	0.265
120	0.405	0.404
160	0.543	0.530
180	0.600	0.610
200	0.676	0.669

Appendix C

The PAS standard curve

The porcine gastric mucin powder was used to obtain a standard curve. a small preweighed amount was dissolved in extraction buffer and brought to volume with water over successive dilutions to give a concentration of 0.2 mg,mucin/ml.

Appendix D

Staining procedure for the silver-based gel electrophoresis colour development system.

The $AgNO_3$ gel development system based on the method of Sammons et al., (1981).

SCHEME

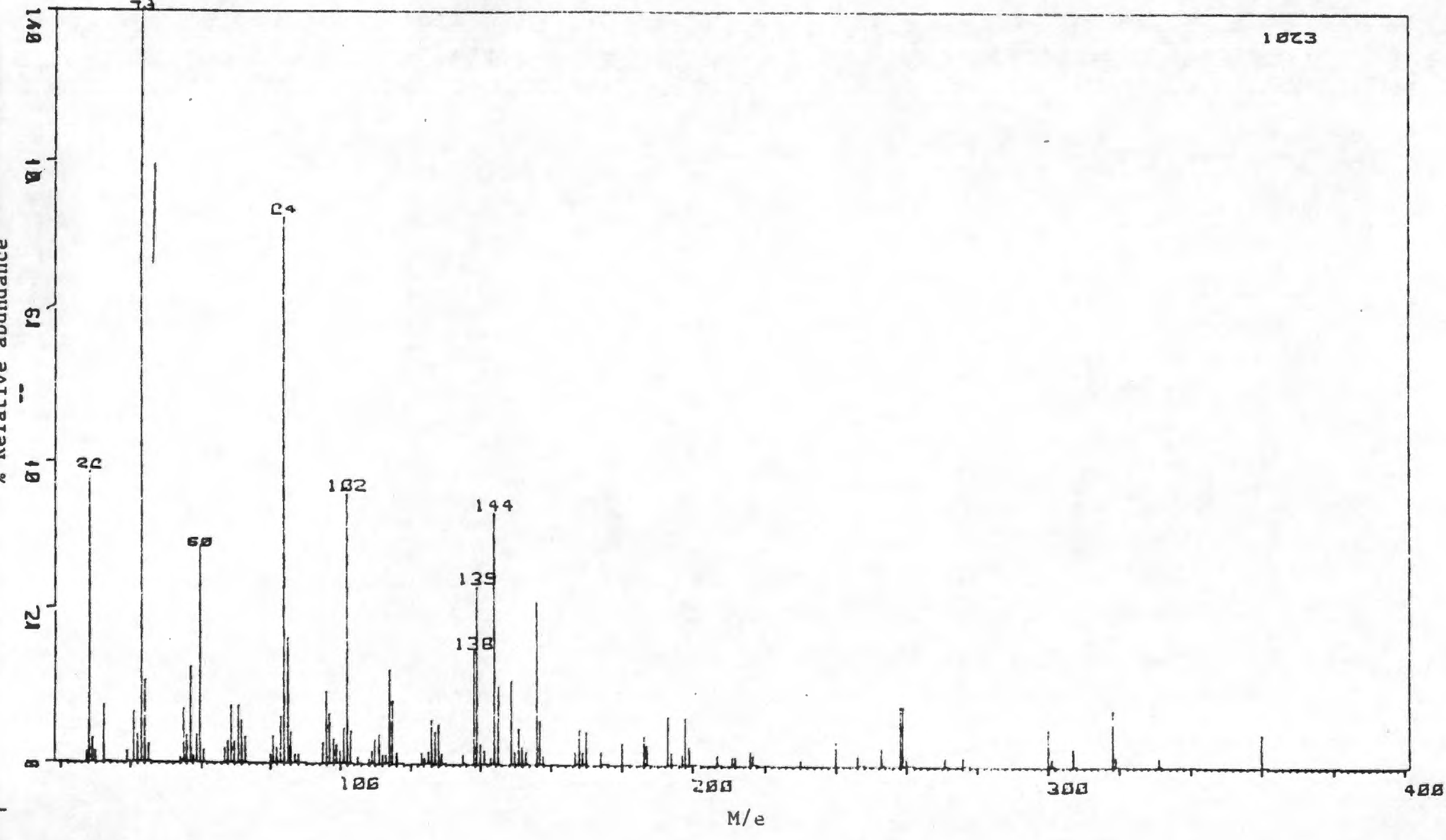
STEPS	SOLUTIONS	DURATION OF AGITATION
Fix	50% EtOH 10% CH_3COOH	2 hours or more
Wash	50% EtOH 10% CH_3COOH	2 hours
	25% EtOH 10% CH_3COOH	1 hour x 2
	10% EtOH 0,5% CH_3COOH	1 hour x 2
Equilibration of Gel	$AgNO_3$ (1.9 g/litre)	2 hours or more
Rinse	H_2O	10-20 seconds
Reduce Silver	$NaBH_4$ (87.5 mg/1)	10 minutes
	HCHO (7.5 ml/1)	
	in 0,75m NaOH	
Enhance colour	Na_2CO_3 (7.5 g/1)	1 hour
	Na_2CO_3 (7.5 g/1)	1 hour, store.

Appendix E

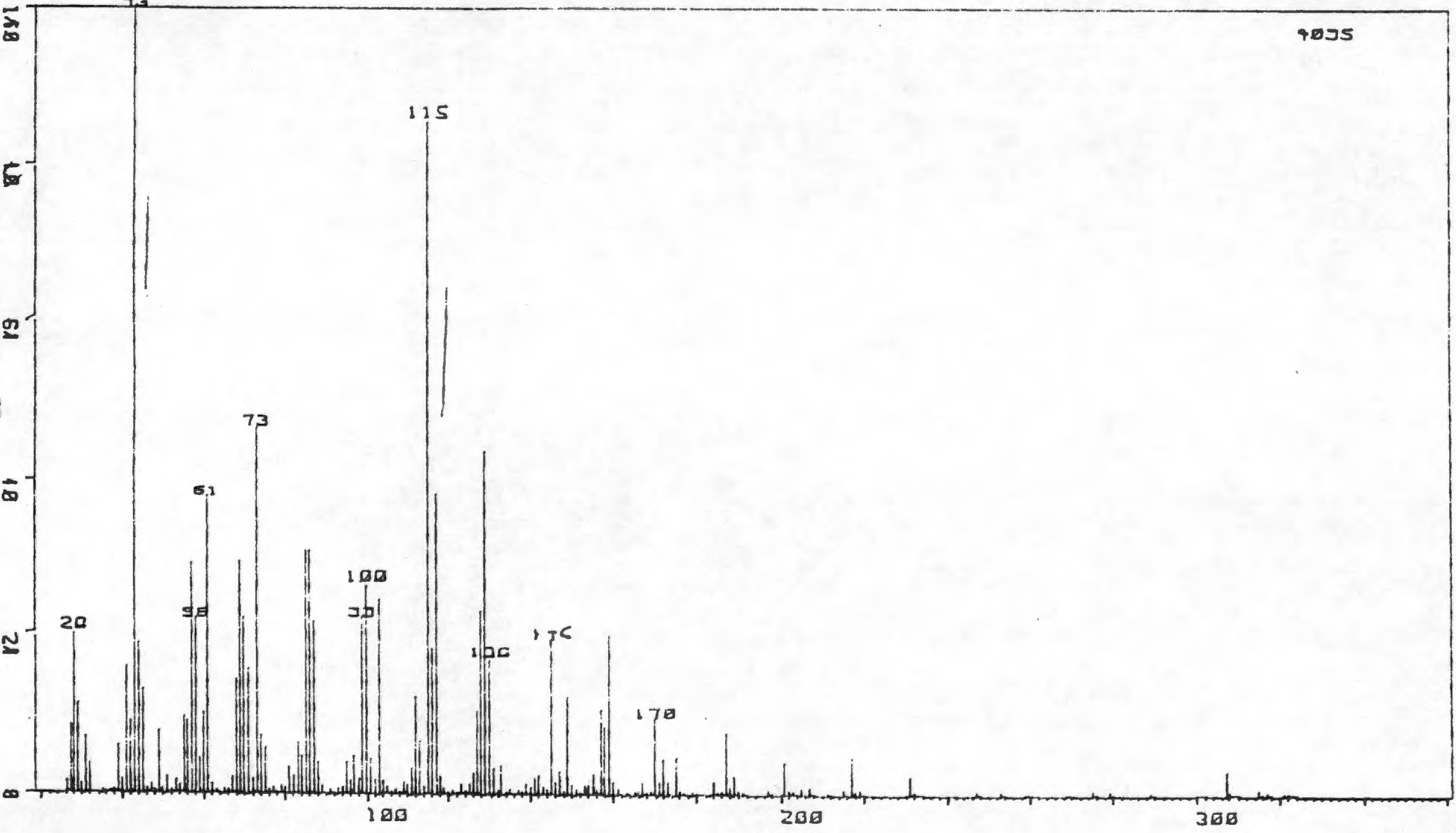
The $AgNO_3$- NH_3 gel development system based on the method of Dubray et al., (1982) and Eschenbruch et al.,(1982)

Step 1.	25% isopropyl alcohol, 10% acetic acid	overnight
Step 2.	7.5% acetic acid	30 minutes
Step 3.	periodic acid $4^{0}C$.	1 hour
Step 4.	glass distilled H_2O, several changes	3 hours
Step 5.	$NH_3 AgNO_3 NaOH$	10-15 minutes
Step 6.	glass distilled water	3 x 5 minutes
Step 7.	Citric acid formaldehyde	until bands appear
Step 8.	H_2O	10-20 seconds
Step 9.	100 ul methylamine in citric acid	overnight
Step 10.	distilled H_2O	Store

APPENDIX F

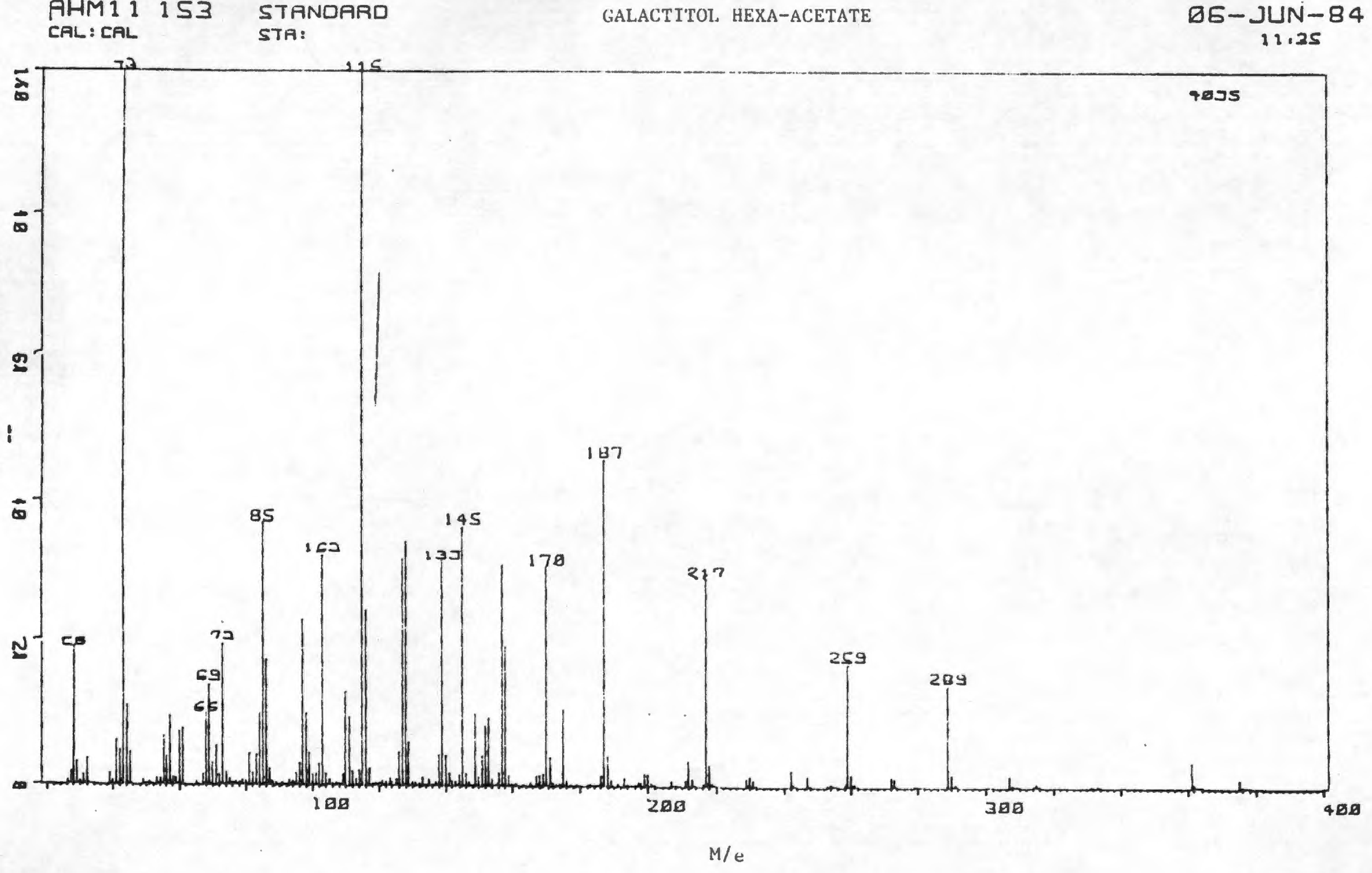


APPENDIX G



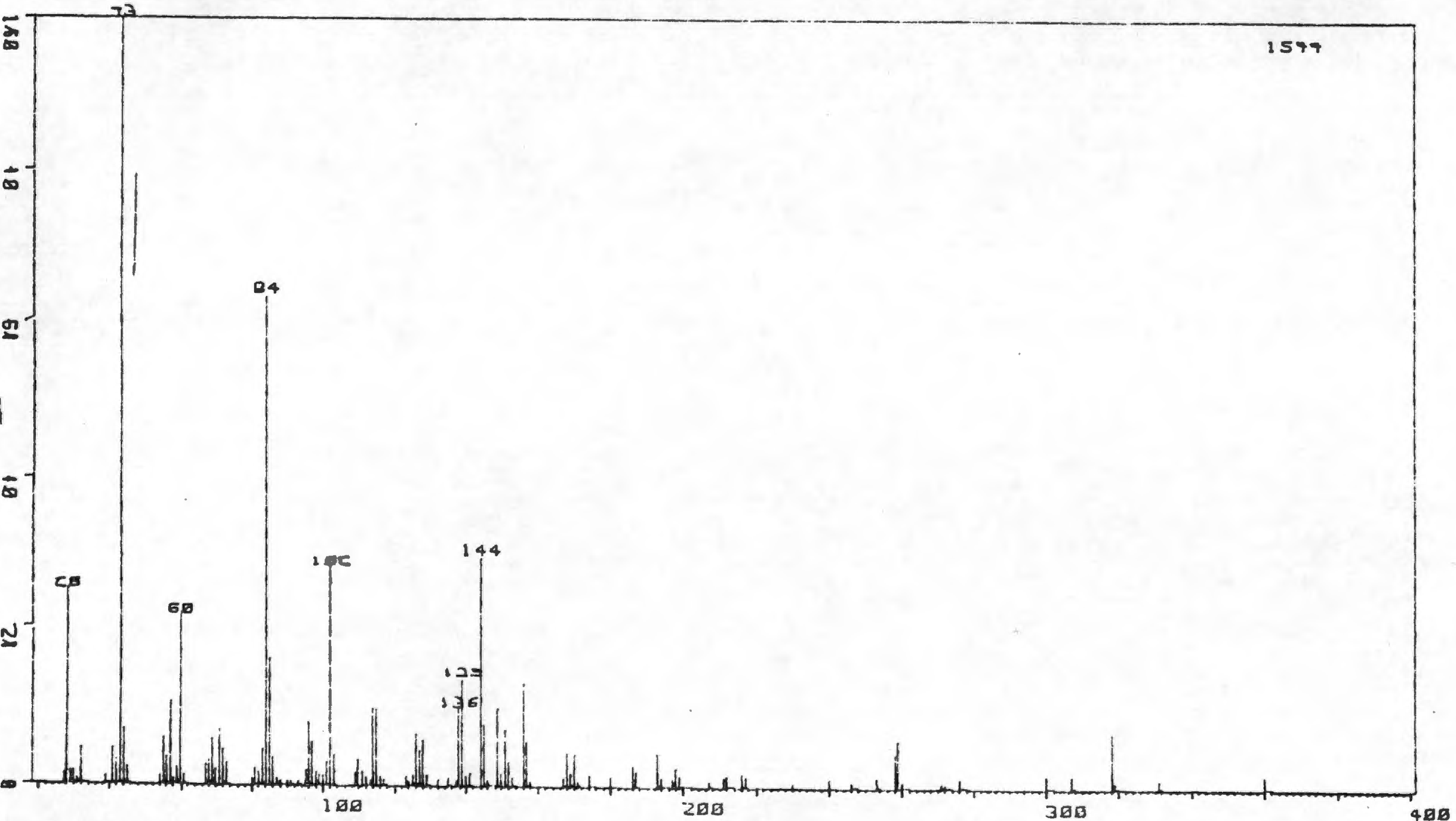
APPENDIX H

HEXITOL HEXA-ACETATE (GALACTOSE ALDITOL ACETATE)

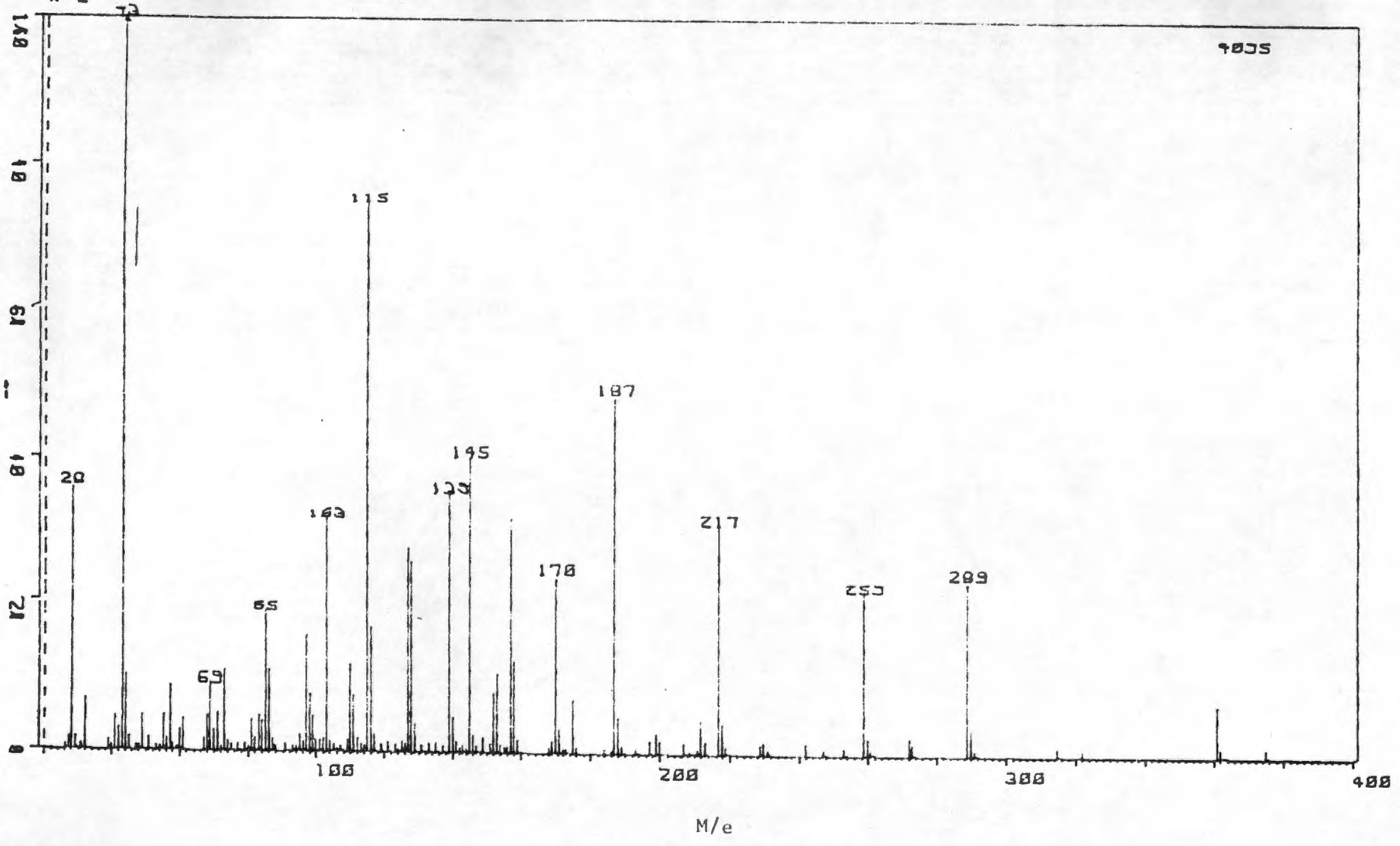


APPENDIX I

PERACETYLATED N-ACETYLHEXOSAMINITOL (GalNAc and GlcNAc)



APPENDIX J



6 MAY 1985

APPENDIX K