# BIOCHEMICAL CHARACTERISATION OF HUMAN GASTRIC MUCIN IN NORMAL AND DISEASED STATES.

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Prof. Rosemary Hickman

I declare that this thesis is my own work.
It is being submitted for the Masters Diploma
in Technology (Medical Technology)
to the Cape Technikon, Cape Town.
It has not previously been submitted for any diploma,
degree or examination at any other institution.
This work was carried out in the Department of Surgery,
UCT Medical School and Groote Schuur Hospital.
The opinions and conclusions drawn are not necessarily those
of the Cape Technikon.

H McLeod ..... 13.4.92 .....

Heather McLeod Date

To Alex, Alexander and Christopher.

## SUMMARY

Gastric cancer, a fatal malignancy, is endemic in the Coloured population of the Western Cape region of South Africa. Diagnosis is based mainly on histologic investigation with patients of either sex being mainly between 40-60 years of age. The extent to which genetic and environmental influences play a role in the aetiology of the disease is unknown.

This study is an attempt to biochemically characterise gastric mucins or mucus glycoproteins, (the main gel forming components of crude mucus scrapings off the mucosal surface), in carcinoma of the stomach (HCA), as compared to those in ulcer disease (HGU), post mortem specimens (PM) and samples obtained from organ transplant donor stomachs (HD). The aim of this study is the development of a diagnostic marker within mucus secretions for the detection of pre-malignant disease amongst the high risk population of the Western Cape region of South Africa.

Mucins were extracted from crude mucus gel scrapings according to a carefully designed technique in which proteolytic inhibitors were used to minimise the possibility of endogenous proteolysis in the laboratory through possible contamination. Two density gradient ultra-centrifugation steps for 48 hours each at 105,000g in caesium chloride, a well established standard isolation procedure for mucins,

gave a yield of pure mucins which fractionated at a density of approximately 1.41g/ml in all groups. These mucins, from the HD, PM, HGU and HCA groups eluted mainly in the included volume of a Sepharose 2B column as broad, polydisperse peaks, suggesting that they were degraded and comprised mainly lower molecular weight PAS positive material in relation to large polymeric gel forming mucin. Sepharose 4B gel chromatography, on the other hand, revealed that this degradation of the mucins was most extensive in the HCA group and decreased in order in the HGU, PM and HD groups. Furthermore mucins in the diseased (HGU and HCA) groups co-fractionated in the caesium chloride gradient with a glycosylated component, the molecular weight of which was shown to be $M_w$55-65kDa by sodium dodecyl polyacrylamide gel electrophoresis (SDS-PAGE). Western blot analysis showed this factor to be closely associated with albumin. The use of different gel stains ($AgNO_3$ and PAS) to highlight proteins and carbohydrates showed that despite this close association, the albumin was clearly distinguishable from the carbohydrate fragment. The complex that formed between albumin and the glycosylated component resisted dissociation in 3.5M caesium chloride, but separated from the mucin in SDS at 100°C for 1-2 mins. The complex was either entirely absent in the control groups (HD and PM) or present in trace quantities of pooled samples of control mucins. Furthermore this albumin-carbohydrate complex eluted in a near total volume of a Sepharose 2B column as a protein (Lowry) positive peak under a carbohydrate (PAS) positive shoulder.

The results indicated that mucins in gastric cancer, though different from those in other groups by virtue of their greater degradation, were similar to mucins from the ulcerated stomachs in that they co-fractionated with an albumin-carbohydrate complex in a caesium chloride gradient. The significance of this complex as a marker for premalignant disease forms part of an ongoing study.

## OPSOMMING

Maagkanker, 'n dodelike kwaadaardigheid is endemies onder die Kleurling bevolking van Wes-Kaapland. Die diagnose berus hoofsaaklik op die histologiese ondersoek van pasiente van albei geslagte, hoofsaaklik tussen 40 en 60 jaar oud. Die mate waarin geneties-en omgewingsinvloede 'n rol speel in die etiologie van die siekte, is nie bekend nie.

Hierdie studie is 'n poging om op 'n biochemiese wyse die maagmusiene of mukusglikoproteïene (die hoof jelvormende bestanddele van ru-mukus wat afgeskraap word van die slymvliesoppervlak) te karakteriseer waar dit voorkom in maagkanker (HCA), en te vergelyk met dié in maagswere (HGU), nadoodse monsters (PM) en monsters verkry van die mae van orgaanskenkers (HD). Die doel van hierdie navorsing is die ontwikkeling van 'n diagnostiese merker in mukusafskeidings vir die uitkenning van voor-kwaadaardigesiekte onder die hoë risiko bevolking van Wes-Kaapland.

Musiene is as uittreksel verkry uit ru-mukus skraapsels deur middel van 'n fyn ontwerpte tegniek waar proteolitiese inhibeerders gebruik is om die moontlikheid van endogene proteoliese as gevolg van kontaminasie in die laboratorium, te verminder. Twee digtheidgradient-ultrasentrifugerings stappe van 48uur elk, teen 105,000g in sesiumchloried, 'n

goed bewese standaard vir die isolering van musien, het 'n hoeveelheid suiwer musien gelewer wat gefraksioneer het in 'n digtheid van ongeveer 1.41g/ml, in alle groepe. Hierdie musien van die HD, PM HGU en HCA groepe het hoofsaaklik geëlueer in die totale volume van 'n Sepharose-2B-kolom as breë, polidisperse pieke, wat gesuggereer het dat hulle gedegradeer was en hoofsaaklik bestaan het uit laer molekulêre gewig PAS positiewe materiaal teenoor groot polimeriese jelvormende musien.

Sepharose-4B-jelchromatografie daarteenoor, het getoon dat hierdie degradering van musien die mees uitgebreide was in HCA en afgeneem het in orde van HGU-, PM- en HD-groepe. Ook het die musien in die siek-(HGU en HCA)-groepe geko-fraksioneer in die sesiumchloriedgradient met 'n geglikosileerde komponent waarvan die molekulêre gewig bepaal was as $M_w$ 55-65kDa deur natrium-dodesal-poliakrilamied-jel-elektroforese (SDS-PAGE). Western-kladanalise het getoon dat hierdie faktor nou-verbonde is aan albumien. Die gebruik van verskillende jelkleurmiddels ($AgNO_3$ en PAS) om proteïene en koolhidrate te merk, het getoon dat ten spyte van hierdie nouverbondenheid, die albumien duidelik onderskeibaar was van die koolhidraatfragment. Die kompleks gevorm deur die albumien- en koolhidraatkomponent het skeiding in 3.5M sesiumchloried weerstaan, maar is van die musien geskei in SDS teen 100°C vir 1-2 minute. Die kompleks was óf heeltemaal afwesig in

die kontrole groepe (HD en PM) óf in spoorhoeveelhede teenwoordig in saamgevoede monsters van kontrole musien. Daarbenewens het hierdie albumien-koolhidraat samestelling geëlueer in 'n bykans totale volume van 'n Sepharose-2B-kolom as 'n proteïen-(Lowry)-positiewe-piek onder 'n koolhidraat-(PAS)-positiewe-skouer.

Die resultate het daarop gedui dat die musien in maagkanker, alhoewel verskillend van dié in ander groepe weens hulle groter afbraak, soortgelyk is aan die musien van mae met ulserasie deurdat hulle gekofraksioneer het met 'n albumien-koolhidraat samestelling in 'n sesiumchloried-gradient. Die belang van hierdie samestelling as 'n merker vir voor-kwaadaardige siekte vorm deel van 'n deurlopende ondersoek.

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## ABBREVIATIONS

| | |
|---|---|
| BCB | Brilliant coomassie blue |
| BSA | Bovine serum albumin |
| CsCl | Caesium chloride |
| DNA | Deoxyribonucleic acid |
| EDTA | Ethylenediaminetetra acetic acid |
| edgu | equilibrium density gradient ultracentrifugation |
| g/ml | grams per millilitre |
| GuHCl | Guanidinium chloride |
| HCA | Human gastric cancer |
| HD | Human Donor |
| HGU | Human gastric ulcer |
| PAGE | Polyacrylamide gel electrophoresis |
| PAS | Periodic acid Schiff |
| mcg/ml | micrograms per millilitre |
| MeSH | 2-mercaptoethanol |
| mins | minutes |
| $M_w$(H/L) | molecular weight (high/low) |
| $NaN_3$ | sodium azide |
| PM | Post Mortem |
| PMSF | Phenylmethyl sulfonyl fluoride |
| NaCl | Sodium chloride |
| NEM | *N*-ethylmaleimide |
| secs | seconds |
| SDS | Sodium dodecyl sulphate |
| w/v | weight per volume |
| O.D. | optical density |

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# CHAPTER 1.

# INTRODUCTION

# 1. INTRODUCTION

## 1.1. Gastric Carcinoma

Gastric carcinoma is the most common cause of cancer death in the world (Parkin et al, 1984). The disease appears to be endemic among the Coloured population of the Western Cape region of South Africa (Dent and Vader, 1981, Wyndham, 1985). No association has been found in this high risk group, between cancer of the stomach and the major histocompatability complex, thereby decreasing the likelihood of a genetic contribution to the disease (Martell et al, 1988). On the other hand, however, elevated levels of nitrites and nitrates, known risk factors in the development of carcinogenesis, have been shown in gastric aspirates of patients with the disease (Coldrey, 1987).

## 1.2. Mucins as markers of malignancy

There has been interest in the use of mucins as diagnostic markers in diseases such as carcinoma of the pancreas (Lan et al, 1987), breast (Price, 1988) and colon (Boland et al, 1982). However, although antigens have been isolated from homogenised specimens of gastric tumours and mucus scrapings, none of these has so far proved to be suitable as tumour markers (Mall et al, 1990). This work describes the characteristics of mucins (purified mucus glycoproteins) in gastric cancer; it is part of a greater study aimed at the development of a diagnostic marker for the detection of gastric premalignancy in a high risk population such as that which exists in the Western Cape region of South Africa.

Previous reports on the characteristics of mucins in gastric cancer have appeared in the literature. There is histochemical and biochemical evidence for changes in the mucins associated with malignant mucosa (Glass and Slomiany, 1977). Both lectins and monoclonal antibodies have been used to detect normal but inappropriate carbohydrate antigens appearing in mucins of gastric adenocarcinomas (Feizi, 1985). In gastric mucins of normal secretor individuals, the terminal blood group antigens are present while antigenic structures typical of non-secretors are masked (Neutra and Forstner, 1987). However when gastric adenocarcinomas develop in the gastric tissues of secretors, the hidden IMa, $Le^a$ and PNL-receptors (peanut lectin) appear as tumour-

associated antigens, presumably due to incomplete synthesis of the H, A, B and $Le^b$ antigenic structures found in normal secretors (Picard and Feizi, 1984). Hakkinen et al, (1968) demonstrated antigenic differences between sulphated glycoproteins from normal and carcinomatous gastric tissue as did Kimoto et al, (1968) who extracted a sulphated glycopeptide from gastric tumours which was absent from normal tissue.

## 1.3. Mucins in patients with gastric carcinoma

In 1978 Bara et al isolated a glycopeptide antigen, M3, from cancerous human gastric mucosa. Whether this sulphoglycopeptide was a component of mucus is uncertain, since papain-digested gastric tissue, and not crude mucus scrapings, was used in the study; also, isolation of the sulphoglycopeptide was by precipitation techniques, now known to be inadequate for the removal of associated contaminant protein non-covalently associated with mucins in crude mucus secretions. An antibody to this glycopeptide was shown to cross react with goblet cells but not with foetal or adult normal gastric mucosa (Bara et al, 1980).

In the present study mucins were isolated from gastric mucus scrapings by density gradient ultracentrifugation in caesium chloride, an established and widely used technique, successful for the isolation and purification of mucins (Creeth and Denborough, 1970). By this technique purified mucins have been shown to be free of other components of mucus (Creeth, 1978). The biochemical characterisation of this purified mucin and an investigation of the possible reproducible generation of a specific mucin antigen in gastric cancer, was the aim of this study.

## 1.4. Mucins in patients with gastric ulceration

Peptic ulceration is a major disease and in spite of a vast amount of research the cause of chronic peptic ulceration is not known (Venables, 1986, Allen and Garner, 1984). The dictum "no acid no ulcer", coined by Schwarz in 1925 (Samloff, 1989), has influenced the direction of peptic ulcer research towards the development of anti-ulcerogenic drugs that inhibit acid secretion. More recently the emphasis of research has shifted to the enhancement of one or more of the endogenous mucosal defence mechanisms eg., mucus and bicarbonate secretion, gastric blood flow and rapid regeneration of the epithelial cell layer. The concept of cytoprotection whereby anti-ulcerogenic agents protect against and heal ulcers without acid inhibition has dominated the literature in the last decade (Robert, 1979). Despite these efforts the problem of ulcer relapse, upon the withdrawal of treatment, remains (Konturek, 1985). The mucus bicarbonate barrier is the first line of mucosal defence against the harsh endogenous aggressors of the gastric luminal environment and the integrity of the adherent mucus gel is necessary for adequate protection of the mucosa (Allen and Garner, 1980, Pearson et al, 1980). Preliminary work has shown that the polymeric gel forming mucins are degraded in human peptic ulcer disease (Younan et al, 1982). In this study, we analysed mucins from gastrectomy specimens of patients with peptic ulcer disease, as a suitable comparison for mucins from patients with gastric cancer.

# CHAPTER 2.

# LITERATURE REVIEW

# 2. LITERATURE REVIEW

Most of the compositional and conformational studies on mucus and mucins have been performed on porcine gastric mucus. The review to follow is largely based on data obtained from studies done on this animal model but has relevance to our human study because of the similarities between human and pig gastric mucus (Allen, 1978, Pearson et al, 1980, Clamp et al, 1983).

## 2.1. Distribution of mucus

Mucus is found in species ranging from the earthworm to man. Research has shown that invertebrates are especially dependent on mucus for survival, using it in a broad spectrum of vital functions. Limpets, earthworms and slugs are protected from dessication by means of a thin external surface barrier of mucus. Here the mucus layer acts both as a lubricant and waterproofing substance to protect the organism from sudden changes in osmotic pressure (Negus, 1967). Mucus facilitates respiration by allowing the exchange of gases through the skin of some invertebrates, eg. eels and slugs and amphibians such as frogs and aquatic toads. Many invertebrates coat themselves with slippery "distasteful" mucus secretions to ward off their predators and mucus secretions of oysters and acorn worms have been shown to contain antibacterial properties (Denny, 1989). Slugs (gastropods) depend on the rheological properties of

their pedal mucus for motility. The mucus alternates between being an adhesive and a viscous fluid due to the forces generated by movement (Denny, 1989). Mucus is also used as a source of nutrition by lower forms of life. This is particularly evident when, for example, a slug crawls over a newly mowed lawn and a caudal mucus plug, used as a defence mechanism, becomes coated with fragments of organic material. This mucus plug, as well as the attached food, is ingested (Denny, 1989).

In higher animals mucus occurs as a sticky, water insoluble secretion which coats the epithelial surfaces of the internal tracts of the body, namely the cervical, respiratory and gastrointestinal tracts. It is produced throughout the length of the gastrointestinal tract by a variety of cell types: oesophageal mucus glands, cardiac, fundic and pyloric glands of the stomach, Brunner's glands of the duodenum, goblet cells of the small and large intestine, and superficial mucus cells found throughout the tract (Allen, 1981). Mucus is also secreted in smaller quantities in the middle ear and by the lacrimal glands of the eye (Fitzgerald et al, 1987) and a primarily soluble mucus is found in human gallbladder bile (Pearson et al, 1982).

In the stomach mucus occurs *in vivo* in three phases namely, the presecreted mucus stored in intracellular vesicles, the insoluble adherent secretion on mucosal surfaces, and the soluble mucus mixed with luminal contents (Ene et al, 1988).

## 2.2. Functions of mucus

In man, mucus is essential to the functioning of the respiratory, genitourinary and gastrointestinal tracts.

Whilst the primary function of mucus in higher animals especially man, is to protect the delicate epithelial surfaces, specialised functions are required by the different organs that produce mucus (Gevers, 1987). As a result, the biophysical properties of mucus, although similar in some aspects, differ according to the role played by the organ concerned (Elstein and Parke, 1977).

### 2.2.1. *Respiratory mucus*

In the respiratory tract, mucus, together with the cilia, is essential for the transport of dust, irritants and bacteria and any other foreign agent from the lungs (Marianne et al, 1987). Mucus lining the luminal surface of airways plays an important defensive role against inhaled airborne particles and chemicals. The protective function is due mainly to the viscoelastic properties of the mucin component of mucus and any abnormality in the structure or amount of these secreted mucins leads to serious pathological problems. Respiratory mucus has been shown to contain immunoglobulins (mainly secretory IgA) and lactoferrin (which chelates iron necessary for the growth of some bacteria) and lysozyme. These constituents are thought to play a role in the anti-viral and

anti-bacterial properties which have been attributed to mucus in some studies (Clamp and Creeth, 1984). Respiratory diseases such as cystic fibrosis and chronic bronchitis are associated with large increases in the mucus secretion (Lopez-Vidriero and Reid, 1978). Elevated carbohydrate levels, implying an abnormal glycosylation of respiratory mucins, are found in patients with chronic bronchitis, emphysema, asthma and chronic obstructive airways disease (Carubelli et al, 1982).

### 2.2.2. *Cervical mucus*

Cervical mucus protects the uterine cavity from invasion by microorganisms and regulates the access of spermatazoa to the site of fertilization in the different phases of the menstrual cycle (Yurewicz et al, 1987). Variations in the properties of cervical mucus during the menstrual cycle are manifested by changes in the viscoelastic properties and water and protein contents of the crude mucus secretion (van Kooij et al, 1980). Mucus that is receptive to sperm is dependent on the relative levels of progesterone and oestrogen. During ovulation, in response to increased oestrogen levels, the water content of the mucus is high and thus the viscosity is low; this facilitates the passage of spermatozoa up the cervical canal. During the luteal phase of the cycle progesterone is dominant, causing the production of less hydrated, more viscous mucus which is impenetrable to the spermatazoa (Wolf et al, 1977).

### 2.2.3. *Gastrointestinal tract mucus*

A general function of mucus is that of lubrication throughout the gastrointestinal tract where it protects the delicate epithelium from the passage of solids, undigested food and faeces through the gut lumen (Allen, 1981).

In the oral cavity mucins appear to confer their protection in association with secretory IgA particularly when salivary mucin and *Streptococci* compete for receptor sites in the buccal epithelium. IgA may coat the bacteria specifically and facilitate aggregation by mucus or phagocytosis by the macrophages and these products are then swept away by the saliva (Neutra and Forstner, 1987).

Intestinal mucus plays an important role in providing a barrier against adherence and invasion by pathogenic organisms. Several methods for this "protection" by mucus have been recognised e.g. aggregation of micro-organisms to facilitate their removal by the flow properties of mucus, interaction of mucus with bacterial enzymes and an increased mucus secretion in response to the presence of toxins and parasites. Bacterial clearance is also aided by mucin binding, coating or clumping of bacteria. These aggregates which are accessible to fluid flow, are unable to adhere to the epithelial surfaces and colonise the cells (Neutra and Forstner, 1987).

Functions assigned to the colonic mucus barrier include protection of the epithelium against the mechanical forces associated with the passage of hard faecal material, provision of a microenvironment and nutrient source for endogenous bacteria, and a physical barrier to invasion by pathogens. Evidence suggests that the mucin carbohydrate side-chains within the adherent colonic mucus layer and invading pathogens compete for cell surface attachment sites, thereby preventing colonisation of the epithelial layer (Allen et al, 1990).

### 2.2.4. *Gastric mucus*

It has long been recognised that the gastric mucosa is capable of resisting acid and peptic digestion, but the mechanisms responsible for this property have not been adequately explained (Rees and Turnberg, 1982). In the stomach, mucus exists either as an insoluble adherent gel on the surface of the mucosa, or as a soluble degraded component mixed with the gastric juices in the lumen. The former is regarded as important for gastric mucosal protection (Venables, 1986), whilst the viscous degraded luminal component is thought to enhance the lubrication of food particles.

2.2.4.1. The adherent gastric mucus gel.

Mucus is secreted as a continuous, viscoelastic water insoluble gel on the mucosal surface of the gastrointestinal tract. Adherent gastric mucus is of variable thickness, ranging between 10 and 400μm (median 80μm) in the rat and 50 and 450μm (median 180μm) in man (Kerss et al, 1982). While the adherent gel is visible in the stomach, duodenum and colon, it is not yet clear how much of the surface gel is present in the small bowel. The adherent gastric mucus gel primarily protects against the natural luminal aggressors, hydrochloric acid (HCl) and pepsin (Allen and Garner, 1984) and the shear forces associated with digestion; to achieve these functions it is imperative that the gel on the mucosal surface is a continuous one.

The question of the continuity of the gastric mucus gel layer is a controversial one. No discontinuities in the mucus layer are observed when mucus thickness is measured in unfixed sections of mucosa using a slit-lamp and a pachymeter (Bickel and Kauffman, 1981), or in the measurement of pH gradients at the mucosal surface (Williams and Turnberg, 1981). Morris et al (1984), found no continuity in the mucus layer using histologically fixed tissue for light and electron microscopy studies. This interpretation has been criticised because standard fixation procedures cause shrinkage and loss of the adherent mucus gel layer (McQueen et al, 1984, Sellers et al, 1987). In subsequent studies

(Tobin and Turnberg, 1989) in which special care was taken for the preservation of the mucus gel layer, for example snap-freezing, a continuous layer of adherent gastric mucus was seen by scanning electron microscopy. Preservation of colonic mucus with mucus antibodies also show a continuous layer of mucus by electron microscopy and without such precautions the mucus layer appears distorted and discontinuous (Bollard et al, 1986).

The idea that mucus on the gastric epithelial surface has a protective function has early origins. In 1954, Hollander proposed a two component barrier for mucosal defence consisting of an alkaline mucus layer on the mucosal surface and a regenerating epithelial cell layer below it. The idea of the role of mucus was developed by Heatley, (1959) who emphasised its function as a mixing barrier by postulating the existence of a pH gradient from the lumen (acidic) to the mucosal (neutral) surface (Allen and Garner, 1984). The subsequent development of electrodes to measure the pH across the mucus layer has favoured the concept of a "mucus-bicarbonate" barrier for mucosal protection (Rees and Turnberg, 1982).

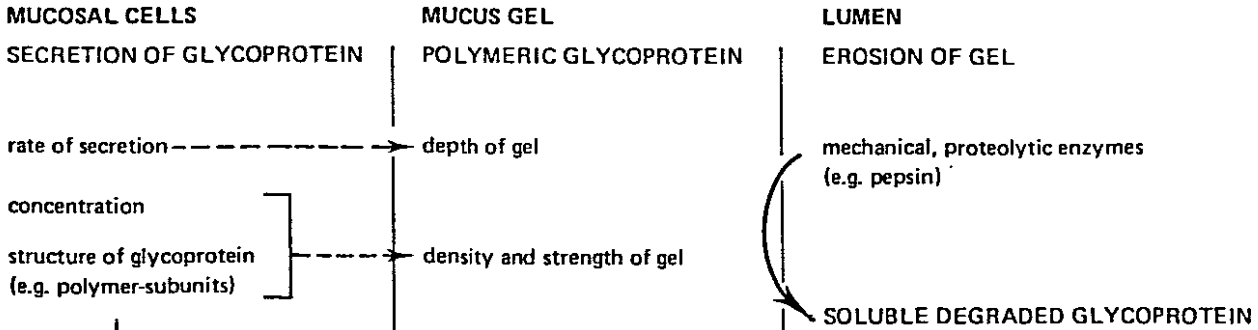



Figure 1

Diagrammatic representation of the factors that influence the dynamic status of the gastric mucus gel on the surface of the mucosa.

(from Allen, 1981)

### 2.2.4.2. Gastric mucus-bicarbonate barrier protection against acid and pepsin (endogenous aggressive factors)

The mucus gel forms an unstirred layer on the gastric mucosal surface separating a layer of $HCO_3^-$ ions from the bulk acid in the lumen. This unstirred layer within the matrix of the mucus secretion will remain despite shear forces associated with the digestive processes ( Allen et al, 1989). A pH gradient has been shown to exist across the mucosa of rat fundus *in vivo* and *in vitro* with a low pH in the lumen and one of near neutrality at the surface of the epithelial cell (Figure 1) (Williams and Turnberg, 1981, Ross et al, 1981). Any acid diffusing through the gel from the luminal side is neutralised by $HCO_3^-$ secreted by the surface epithelial cells (Rees and Turnberg, 1982) and the rising pH towards the epithelial surface would also inhibit the action of pepsin. Williams and Turnberg (1981) showed that the rate of diffusion of hydrogen and sodium ions through pig gastric mucus is delayed to a greater extent than the rate of diffusion through a layer of unstirred water of similar thickness.

The mucus gel is permeable to lower molecular weight molecules up to the size of vitamin $B_{12}$ ($M_w$ 1346) thus acting as a barrier to larger molecules like pepsin ($M_w$~34,500). Pepsin however is able to degrade the gel on the luminal surface (Allen and Garner, 1980). This action does not diminish the thickness of the gel which is continually

replenished by secretion of mucus from the epithelial cells. While the unstirred layer of mucus on the surface of the mucosa provides adequate protection against pepsin, acid and shear, its breakdown by an excess of pepsin perhaps, and a subsequent imbalance in secretion and degradation, would impair the function of the gastric mucus barrier (Allen et al, 1989).

### 2.2.4.3. Gastric mucus protection against exogenous aggressive factors

It has been documented that certain ulcerogens e.g. ethanol, non-steroidal anti-inflammatory drugs (NSAID's), hypertonic saline and bile salts permeate the gastric mucosa easily (Allen et al, 1986). As mucus offers no protection against such agents, the underlying epithelial cells are destroyed (Robert, 1979). While ethanol dehydrates the mucus layer, it and other agents can cause different kinds of damage including vascular disruption, haemorrhage and visible surface damage. If mucosal damage is acute and confined to the epithelium with the basal lamina remaining intact, the damaged epithelium is rapidly restored by replacement of necrotic cells with preformed cells migrating from the gastric pits (Lacy and Ito, 1984). Histologic studies show that with more severe damage following an ethanol insult there is a massive release of gelatinous material, which forms a cap over the re-epithelialising mucosa (Sellers et al, 1987). This gelatinous coat is quite different from the

native mucus and immunoperoxidase stains have shown that this layer is basically a fibrin gel mixed with necrotic cells and remaining mucus (Sellers et al, 1987). Histological analyses have shown that the mucoid cap provided a protective layer, allowing for restitution of the damaged epithelium and protection of the healed mucosa from further damage by some exogenous factors (Wallace and Whittle, 1986).

### 2.2.5. *Mucus in the gallbladder and ear*

The functions of gallbladder mucus are as yet unclear (Pearson et al, 1982) but it most likely provides protection against surface active chemicals e.g. bile salts which destroy the epithelial lining of the gallbladder. Recent research strongly suggests that gallbladder mucus enhances formation of gallstones (Smith, 1990); the hydrophobic binding properties of gallbladder mucin act as nucleation centres for gallstone formation (Gevers, 1987, Pearson et al, 1982). Smith (1990) suggests that retention of microscopic crystals in the mucus gel of the gallbladder may permit the growth of these crystals to a size which is not compatible with physiological flushing from the gallbladder lumen into the small intestine after a meal.

In the normal state, mucus is presumed to be secreted into the middle ear to facilitate the clearance of particles and foreign bodies via mucociliary clearance down the Eustachian tube (Fitzgerald et al, 1987). Hypersecretion of this mucus is associated with conditions like otitis media.

## 2.3. Composition of crude mucus

Generally the crude mucus secretion is a complex mixture of water (95% or more), mucins (0.5-1%), inorganic ions (present at the same concentration as found in plasma), secreted and transuded proteins (0.5-1%), nucleic acids, bacteria, dead cells, partly digested food particles, digestive enzymes, lysozymes and bile salts. Proteins present in mucus secretions include secretory IgA and lactoferrin whilst albumin, IgA and IgG are examples of the transuded proteins found in mucus. Lysozymes destroy bacterial cell walls and lactoferrin inhibits the growth of iron dependent bacteria; these properties suggest that they play a protective role in the native secretion (Creeth, 1978). It has been reported that serum albumin passes into the lumen in normal conditions, and in excessive amounts in gastric cancer (Creeth, 1978 ). Mucus preparations from the stomach and the small intestine have been shown to contain between 5% and 20% lipids depending on the isolation technique used (Fahim et al, 1983). Studies on purified intestinal mucins, showed an absence of covalently or non-covalently bound fatty acids (Mantle and Forstner, 1986) whilst other researchers have reported that lipids can protect gastric mucin from proteolytic degradation (Slomiany et al, 1984).

Genitourinary and gastrointestinal tracts normally contain little or no nucleic acids, whilst purulent sputa contains increased amounts of nucleic acids. In patients with cystic

fibrosis, DNA concentrations of 4mg/ml have been reported (Creeth, 1978). Mall et al (1987) showed that small amounts of contaminant DNA can markedly influence the viscosity of pig duodenal mucus glycoprotein preparations. An important component, though minimal in content, is intrinsic factor which is necessary for the absorption of vit $B_{12}$ (Allen, 1981). Mucus glycoproteins (mucins) which impart gel forming properties to the mucus secretion (Hollander, 1954), constitute between 1 and 10% by weight of the gel. The concentration of mucins in the gel varies according to the source of the mucus, pig gastric and pig duodenal mucus containing approximately 50mg/ml, pig small intestinal mucus 20mg/ml and pig colonic mucus 30mg/ml (Bell et al, 1985).

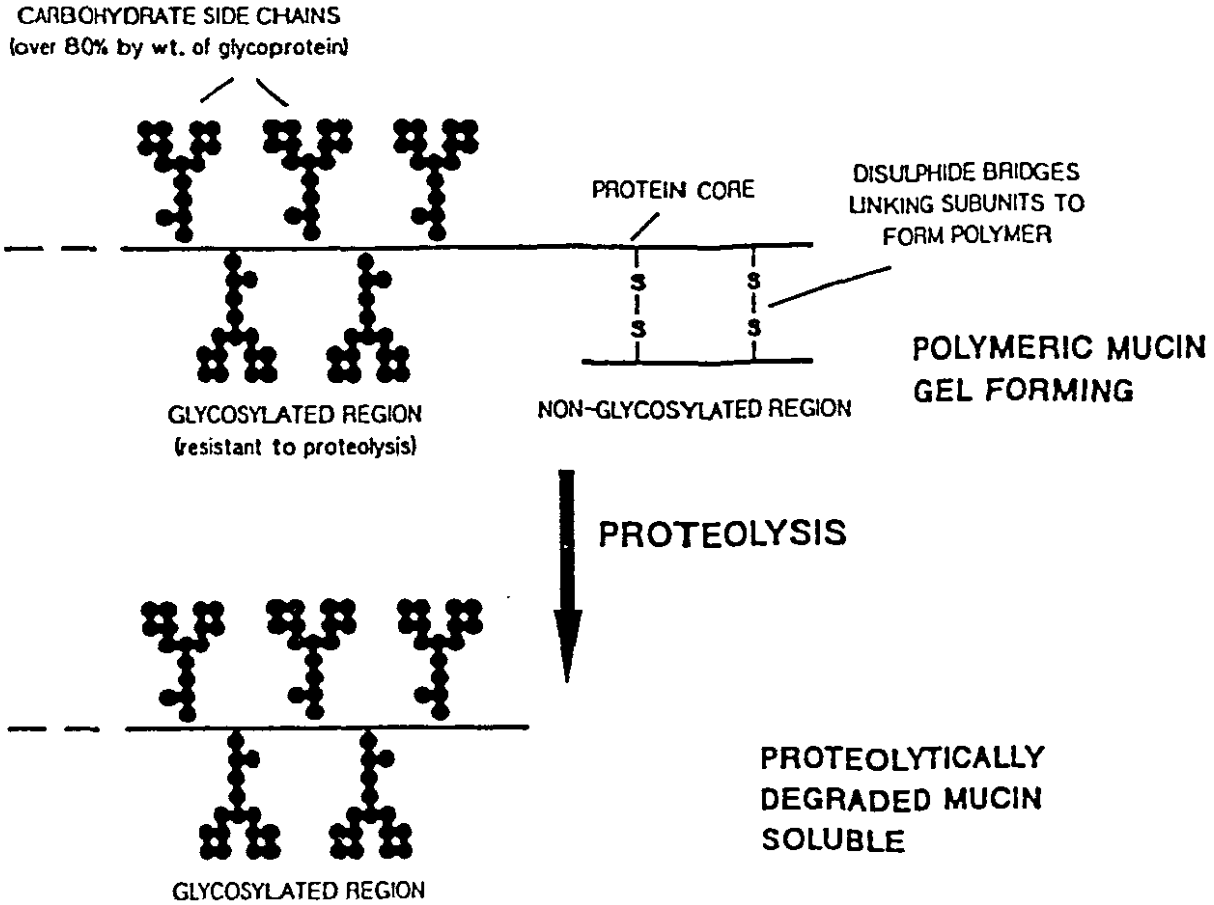



Figure 2

Diagrammatic representation of a mucus glycoprotein (mucin) subunit. The subunits between the non-glycosylated regions of the protein core are linked through disulphide bridges. (from Allen, 1989)

## 2.4. Structure and composition of mucins

Mucins are high-molecular weight glycoprotein (protein-polysaccharide) complexes which polymerise to form an insoluble gel in water, their reported sizes ranging between 2 and 44 million daltons depending on the isolation techniques used (Allen et al, 1976, Carlstedt and Sheehan, 1984). The composition of mucins is complex but there are basic features common to all mucins despite varying origins. These are (a) a high carbohydrate content: 70-80% by weight (b) a low protein content: 18-22% by weight and (c) ~5% ester sulphate. Polymeric mucins consist of subunits which are made up of protein cores with glycosylated and non-glycosylated regions. The latter has cysteine residues which allows for disulphide bonding between subunits to form polymers. The glycosylated regions are "covered" with carbohydrate side chains and this characteristic has been likened to a bottle brush, where the bristles are the oligosaccharides and the wire supports the protein core (Figure 2).

### 2.4.1. *The carbohydrate side-chain of mucins*

A typical complement of sugars found in a mucin molecule comprises *N*-acetylgalactosamine, *N*-acetylglucosamine, fucose galactose and sialic acids. Mucus glycoproteins do not contain either uronic acid, which is a sugar characteristic of the proteoglycans of connective tissue, or mannose, which is found in serum glycoprotein and some membrane

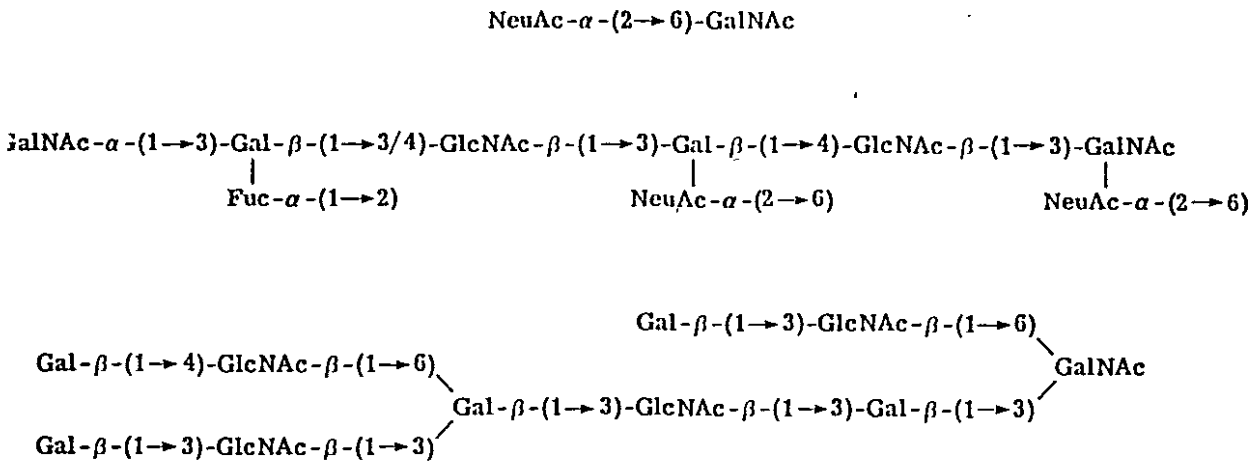


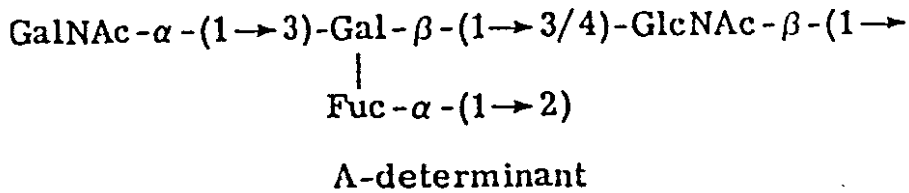

Figure 3

Diagram of mucin oligosaccharides which are joined through *N*-acetylgalactosamine in an α-linkage to the hydroxyl group of serine or threonine residues in the protein core to form O-glycosidic bonds.

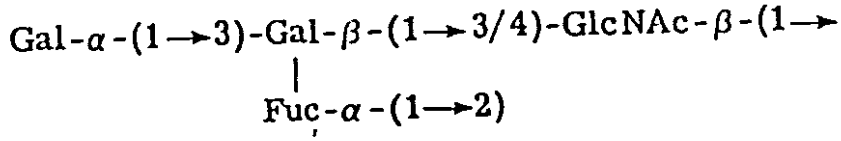
(from Carlstedt and Sheehan, 1985).

glycoproteins (Allen, 1981). There is a distinct difference in the composition, structure and size of oligosaccharides between secretions from different species. Differences in molar proportions (composition) e.g. 9.8 mol% of *N*-acetylgalactosamine compared with 35.4 mols% of galactose could be due to differences in the ABH(O) and Lewis blood groups or it could be species specific (Neutra and Forstner, 1987). The oligosaccharides may be 2 sugars in length (sheep sub-maxillary mucin) (Gottschalk et al, 1972), 4 sugars in length (pig submaxillary mucin) (Eckhardt et al, 1987), or may be a complex mixture of 19 sugars (pig and human gastric mucin), (Slomiany et al, 1978, Schrager and Oates, 1974).

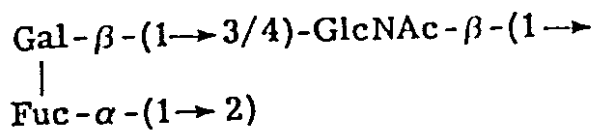
There are common structural patterns for the carbohydrate side-chains (Figure 3). Mucin oligosaccharides are joined to the protein core through *N*-acetylgalactosamine in an $\alpha$-linkage to the hydroxyl oxygen of serine or threonine residues, forming O-glycosidic linkages. This link occurs at every third or fourth amino acid residue or sometimes with adjacent serine and threonine residues. *N*-acetylgalactosamine is always found at the reducing end of the chain (Carlson, 1977). The only other position where *N*-acetylgalactosamine occurs in the oligosaccharide chain is in a terminal position. The backbone of the oligosaccharide chain has alternating residues of beta linked galactose or *N*-acetylglucosamine. In human and pig gastric mucins, the sugar linking the chain to the *N*-acetylgalactosamine at the reducing end is $\beta$-galactose while in human and rat colonic




A-determinant




B-determinant




H-determinant

Figure 4

Terminal sugar sequences of chains exhibiting determinants of the ABH(O) blood group system.

(from Carlstedt and Sheehan, 1985).

mucins it is β-*N*-acetylglucosamine. Sialic acids are normally found in terminal positions at the non reducing ends while ester sulphates are located internally on the longer oligosaccharides linked to a galactose or *N*-acetylglucosamine residue. Many carbohydrate side-chains carry a negative charge due to the presence of sialic acid residues and ester sulphates.

There are certain common antigenic features between different mucins determined by the structure of the non-reducing ends of the oligosaccharide chains e.g. the ABH(O) and Lewis blood group antigenic determinants on the surfaces of the erythrocytes are the same as the structures of the terminal portions of the oligosaccharide side-chains of mucins (Figure 4). The specific antigen activity is reflected in the terminal sugar. An A secretor has N-acetylgalactosamine at its non-reducing end, a B secretor has galactose at its non-reducing end and an H secretor (O blood group) ends with fucose-2 galactose sugars (Carlstedt et al, 1985).

2.4.2. *The protein core of mucins*

Several studies on gastrointestinal mucins have shown that the protein core is made up of two regions, each with its own amino acid composition (Scawen and Allen, 1977, Schrager and Oates, 1971). About 35% constitutes the so-called "naked" region and consists of amino acids typical of a globular protein but with disulphide bridges. Some of the

amino acids in this minor region of the protein core are alanine, glycine, valine and cysteine, the latter forming disulphide bonds with other glycopeptide monomers or with a "link" peptide that bridges two or more glycopeptides during the formation of high molecular weight mucin polymers (Fahim et al, 1983, Pearson et al, 1981). The major part of the protein core is glycosylated and is rich in serine and threonine (attachment sites for the oligosaccharides) and proline, thought to be necessary to achieve a conformation that will permit the close packing of the carbohydrate chains. It is protected from proteolysis by the carbohydrate sheath while the non-glycosylated region can be digested away by a variety of proteinases (pepsin, trypsin, papain and pronase) (Scawen and Allen, 1977).

### 2.4.3. *Structural differences in mucins from different regions*

Colonic mucins are generally larger than most other mucins ($M_w$ $15 \times 10^6$) and tend to have an amino acid profile more similar to gastric mucins than to intestinal mucins (Allen, 1983). Some mucins possess a protein component, the so-called "link" protein that is part of the polymeric structure but easily separated from the polymer by reduction of the disulphide bridges. Pig and human gastric mucins have a link protein of $M_w$70,000 (Pearson et al, 1981), human and rat small intestinal mucins have link proteins of $M_w$118,000

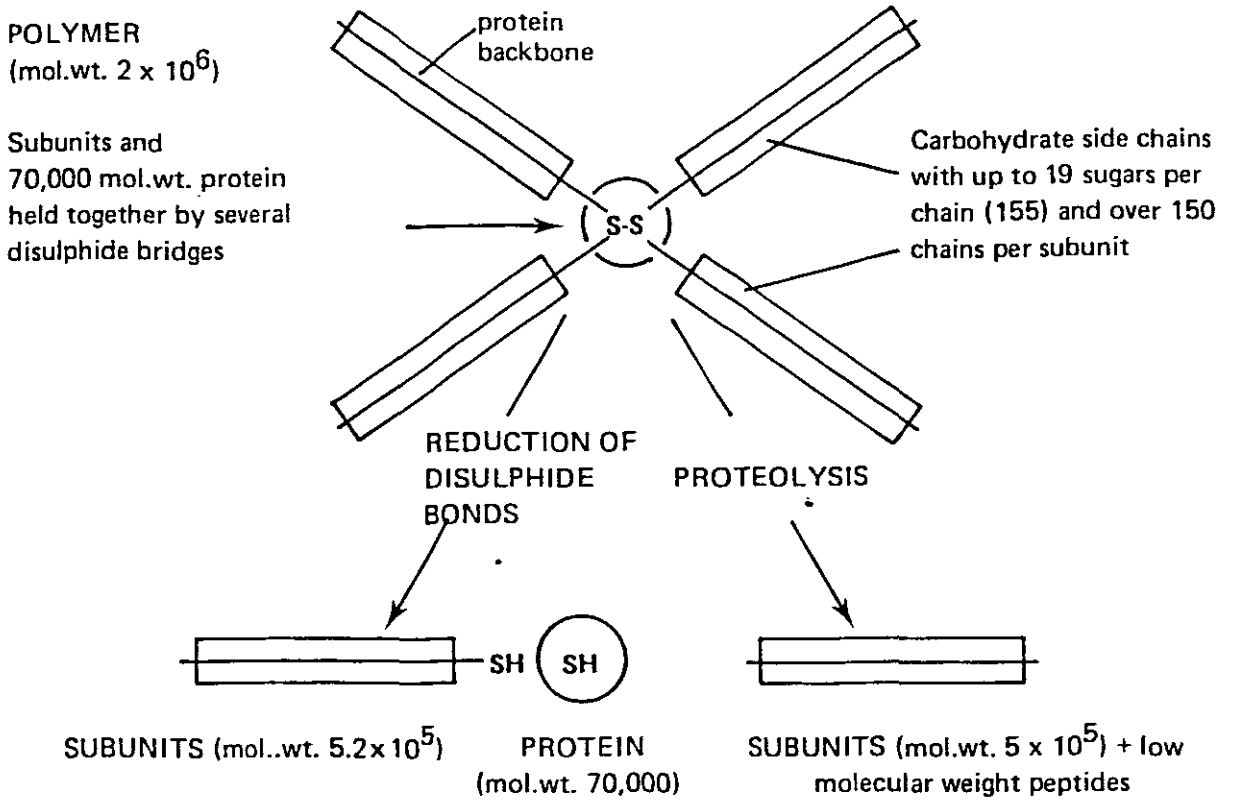



Figure 5

Digrammatic representation of the "windmill" model proposed by Allen, (1978).

(Fahim et al, 1983) while colonic mucins do not have a link protein. Both respiratory and cervical mucins have a polymeric structure whereby subunts are linked together by disulphide bonds although details of the size of subunits are not as well defined as for gastrointestinal mucins (Roberts, 1978). Salivary mucins differ from gastric mucins in that they have a polymeric structure formed from non-covalent, ionic dependent, associations of subunits, although disulphide bridges still play an important role in their conformation (Sellers and Allen, 1984).

Because mucins are made up of monomers that vary in size and composition they are intrinsically heterogenous and polydisperse in nature. The polydispersity of the polymeric mucin is due to differing mucin monomers. Microheterogeneity occurs at the level of synthesis, but can occur in the gut where proteolytic enzymes hydrolyze parts of the protein core.

### 2.4.4. *Structural models proposed for the mucin molecule*

There are two schools of thought regarding the structural and conformational model for mucins. The so-called "windmill" model has been proposed by Allen (1978) (Figure 5) whereby mucins are highly expandable molecules which interact at their surfaces within a solvent domain to take on a spheroidal shape. The mucins are composed of 4 subunits ($M_w 5x10^5$) (Figure 5) which have polymerised to form the

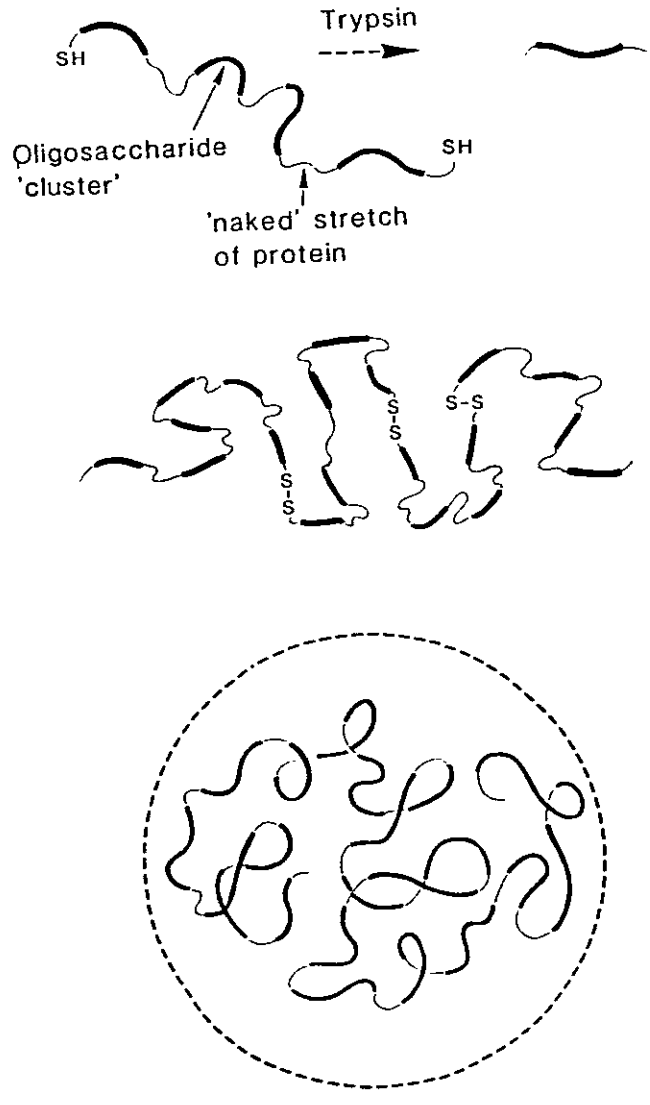



Figure 6

Diagrammatic representation of the linear flexible model proposed by Carlstedt and Sheehan, (1984).

native glycoprotein by disulphide bonding of each subunit to a link protein of Mw70kDa. When mucins are treated with 2-mercaptoethanol (MeSH) or proteolytic enzymes the link protein is released or digested as shown by polyacrylamide gel electrophoresis, effectively separating the subunits (Pearson et al, 1981).

The size of the gastric mucin is significantly affected by the isolation procedures employed. The "windmill" model has been isolated in 0.2M NaCl without proteolytic inhibitors and is substantially smaller than the alternative proposed model of Carlstedt and Sheehan (1984). Evidence that the subunits are centrally joined is based on N-terminal analysis whereby the same N-terminal amino-acids isoleucine, valine and phenylalanine, were found before and after trypsin treatment (Scawen and Allen, 1977, Burgess and Allen, 1983).

An alternative model for porcine gastric mucus is that of a long peptide, containing several (4-5) glycosylated regions alternating with naked stretches, that make up one subunit (Figure 6) (Carlstedt and Sheehan, 1984). The subunits are linked end to end via S-S bonds to form a long partially coiled thread with some areas (naked regions) more flexible than others (glycosylated regions). According to this model the subunits have molecular weights of $2\text{-}3\times10^{6}$. Each subunit can be digested by trypsin into fragments ($M_w\sim3.8\times10^{5}$) often referred to as T-domains and mainly carbohydrate in nature.

In either case the nonglycosylated protein cores are sensitive to proteolysis by proteinases while the glycosylated regions are protected from such proteolytic attack.

## 2.5. Extraction and purification of mucins

In order to understand how the functional properties of mucus are related to its structure, the mucinous component has to be isolated. The true size of glycoprotein and the size of its constituent subunits differ between investigators as do the techniques used for the extraction and purification of the molecules (Carlstedt and Sheehan, 1984, Allen et al, 1989). The main objective is to free the mucin component of non-covalently bound protein and nucleic acids, at the same time retaining the mucin molecule in as near a native state as possible.

### 2.5.1. *Solubilisation of mucus*

The two most effective methods for solubilizing mucus gels are (a) reductive cleavage of the disulphide bridges with thiol reagents, or (b) proteolysis with either endogenous or added proteolytic enzymes (Allen, 1981). Mucin "fragments" obtained after reduction are generally referred to as subunits. Subsequent proteolytic digestion of subunits affords glycopeptides (T-domains) corresponding to the glycosylated regions of the macromolecules (Carlstedt and Sheehan, 1984). Degraded glycoproteins of low molecular weight are suitable materials for investigations of the oligosaccharide structure but their properties are not representative of the larger undegraded mucins as they have lost much of their viscous and gel forming properties

(Creeth, 1978, Scawen and Allen, 1977).

High-speed homogenisation in non-denaturing solutions (0.2M NaCl,) or slow agitation in denaturing solutions (urea, guanidinium chloride) are two methods widely used for the extraction of mucins (Allen, 1981, Carlstedt and Sheehan, 1984). Pig gastric mucus is completely solubilised in 0.2M NaCl with brief homogenisation (60 secs). This method yields mucins of $M_w 2x10^6$ with full gel forming properties, consistent physical and chemical properties and a size that is readily affected by proteolysis (Allen et al, 1989). On reduction with 0.2M 2-mercaptoethanol these mucins split into subunits of $M_w 5x10^5$. Gastric mucins isolated in guanidinium chloride and proteolytic inhibitors are of a much larger size - $M_w \sim 44x10^6$ (Carlstedt and Sheehan, 1984). These mucins are heterogenous in size and have no gel forming properties, precipitating from solution at gel forming concentrations. The large size of mucins, isolated in guanidinium chloride, with or without proteolytic inhibitors could be due to aggregation caused by the denaturing effect of guanidinium chloride (Snary et al, 1974). This is borne out by studies done by Hutton et al, (1983) where it was shown that on heating (100°C in 1% SDS), large mucins prepared in guanidinium chloride with or without proteolytic inhibitors, dissociated into smaller sized entities. The latter were of the same size as mucins isolated in 0.2M NaCl suggesting that the original large molecules were aggregates of mucins of size $M_w 2x10^6$ joined by non-covalent bonds.

### 2.5.2. *Purification of mucins*

Mucus glycoproteins are complex molecules of large molecular size which can be isolated by permutations of three widely used methods: gel chromatography (size), ion exchange chromatography (charge) and equilibrium density gradient ultracentrifugation (buoyant density) (Allen, 1989). While gel and ion exchange chromatography are widely used, a common disadvantage is that non-mucin proteins which are strongly non-covalently bound to the mucins co-fractionate with them in a variety of isolation techniques. While non-covalent bond breaking reagents e.g. guanidinium chloride and urea, can be used to separate the mucins and contaminant protein, the most effective way to isolate mucins is to purify by means of equilibrium density gradient ultracentrifugation in caesium chloride (Starkey et al, 1974). The ionic strength of the salt (3.5M in the case of CsCl) counteracts the non-covalent interactions between molecules e.g. protein-glycoprotein. The purity and homogeneity of the isolated mucin can be determined by measuring protein content, demonstrating a unimodal peak on gel filtration or SDS-PAGE (Pearson et al, 1981). Electrophoresis with or without SDS is particularly good for assessing contaminant protein content (Forstner et al, 1973). If uronic acid or mannose were detectable in these preparations, it would be indicative of proteoglycan or serum glycoprotein contamination (Allen, 1981).

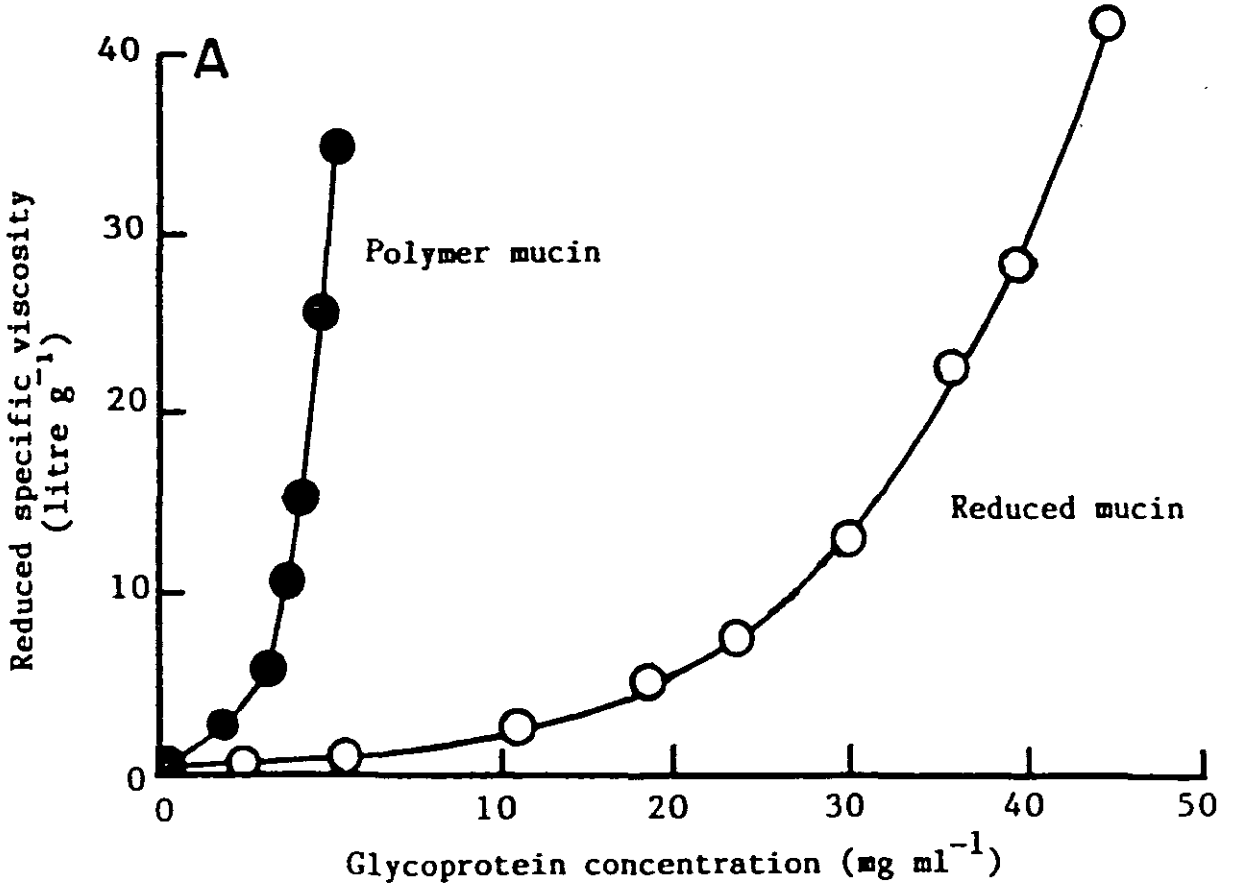



Figure 7

Diagram showing the increase in viscosity of polymeric mucin (●) and reduced mucin (○) with increasing glycoprotein concentrations. As the mucin concentration increases, the viscosity rises asymptotically until the solution assumes viscoelastic gel-like properties and a gel is formed (~50mg/ml for pig gastric mucin).

(from Allen, 1989).

## 2.6. Viscous and gel forming properties of mucus

The adherent mucus gel found on the gastric mucosal surface has a typical viscoelastic nature (Bell, et al 1984). The viscous or liquid properties are shown by its abilities to flow by gravity and to anneal. The elastic or solid properties are exhibited by its resistance to deformation and the ability to adhere. The component that determines these properties of mucus is the polymeric mucus glycoprotein. This is demonstrated by the *in vitro* formation of a gel which has the same mechanical properties as the native gastrointestinal secretions when purified mucin is present at concentrations the same as those found *in vivo* (Bell et al, 1985).

The impenetrability of the mucus gel is dependent on the concentration of the mucin molecules within the total mucus secretion. Gel formation can be monitored *in vitro* by measuring the viscosity of a solution as the mucin is concentrated (Allen, 1981). As the mucin concentration increases, so does the viscosity, which is linear up to a mucin concentration of ~20mg/ml. The rise in viscosity thereafter is asymptotic, rising sharply until a gel is formed (Figure 7). A model for gel formation shows that mucin molecules are highly expanded, hydrated and roughly spherical in shape and that at 20 mg/ml they fill the entire solvent domain. Above this concentration the mucin domains begin to overlap, the intermolecular interactions increase

and gel formation takes place (Allen et al, 1976). Pig gastric mucin concentrates around 50mg/ml *in vivo*. The higher the glycoprotein concentration the greater the degree of interpenetration and the more stable the gel will be. This arrangement within the gel matrix creates the unstirred layer which prevents $HCO_3^-$ secreted by the epithelial cells from mixing with the luminal HCl.

The strong negative charge of gastrointestinal mucins due to bound ester sulphate and sialic acid residues determines the degree of expansion in solutions of varying electrolyte concentrations (Snary and Allen, 1971, Allen, 1978). In water or low salt concentration solutions the viscosity of mucins is greatly increased. The negatively charged residues repel each other in the absence of cations resulting in expansion of the molecules in solution (Allen, 1977). Forstner et al (1973) showed that this effect could be removed if the negatively charged residues were removed, in this case with neuraminidase. Addition of calcium to rat small intestinal mucins decreases the viscosity and causes clumping, a condition noticed in mucins isolated from secretions of patients with diagnosed cystic fibrosis (Allen, 1981).

Many studies have been done to determine the effect of mucus degradation on the viscosity measurement of a mucus gel. Proteases degrade mucins by nibbling at or digesting the naked protein region of the mucins and mucolytic agents

reduce mucin molecules by splitting the disulphide bridges (Snary et al, 1970). These degraded forms of mucins exhibit reduced intrinsic and specific viscosities, suggesting that polymeric mucins are essential for gel formation. At a given mucin concentration, the specific viscosity of reduced mucin is markedly lower than that of polymeric mucin. Carbohydrate chains of mucins are of the utmost importance to a strong highly impenetrable gel (Allen, 1981), with the intermolecular oligosaccharides interdigitating with each other to create a denser gel which is stable and less flexible. It has been reported that non-covalently bound proteins and lipids in the total mucus secretion enhance the viscosity of the gel (Creeth, 1978, Sarosiek et al, 1984). However purified intestinal mucins are free of covalently bound lipids (Mantle and Forstner, 1986) and the removal of non-covalently bound proteins from mucus of other regions has consistently increased the viscous nature of the mucins (Mantle and Allen, 1981).

## 2.7. Biosynthesis of mucins

Mucins are produced and secreted from a variety of cell types e.g. surface epithelial cells of the salivary glands, oesophagus and stomach, goblet cells of the small and large intestine, Brunners glands in the duodenum and mucus cells of the gall bladder and pancreatic ducts (Neutra and Forstner, 1987).

There has hardly been any experimental evidence regarding the biosynthesis, storage and secretion of mucins from their relevant cell types. Studies to date have been based on the assumption that synthesis of the protein core and its subsequent glycosylation occurred as in the conventionally described manner for glycoproteins (Phelps, 1978), whereby the protein core is synthesised in the rough endoplasmic reticulum (RER) and its glycosylation takes place in the Golgi apparatus (Allen, 1981, Neutra and Forstner, 1987).

Antibodies raised to salivary gland apomucin failed to react with the products of the synthetic process in the Golgi apparatus, suggesting that glycosylation resulted in the epitopes of the protein core being masked (Deschuyteneer, 1988). However the initial O-glycosylation i.e. the linkage of *N*-acetylgalactosamine to serine or threonine, could be located in a late RER compartment.

A recent detailed study by Dekker and Strous, (1990) using inhibitors of cellular processes showed that mucin precursors (protein cores) of each subunit oligomerise in the RER by forming intermolecular disulphide bonds, resulting in a mixture of di- and trimers of molar ratio 3:2. Using tunicamycin, an inhibitor of glycosylation, it was shown that *N*-glycosylation of the precursor is necessary for efficient oligomerisation (Dekker and Strous, 1990). The function of the *N*-linked glycans is thought to be in protein folding to facilitate correct intra- and intermolecular disulphide bond formation of the mucin precursors.

This oligomerisation, an ATP independent mechanism confined to the RER, was also shown to be independent of the initial O-glycosylation process, which occurred in a late RER or early Golgi compartment as shown by the use of Brefeldin A, a fungal metabolite which inhibited RER to Golgi transport and also induced the presence of glycosyltransferases to the RER. Extension of the oligosaccharide and maturation of the mucin (i.e. sulfation) occurred in the medial and trans-Golgi compartments respectively (Dekker and Strous, 1990).

Completed mucin macromolecules are packaged in supra nuclear secretory vesicles and migrate towards the apical surface of the cell from where they are extruded.

## 2.8. Mechanisms of secretion of mucus

Mucus secretion is a complex function of the gastric epithelium. The mucus secreting cells are highly differentiated, a property responsible for variations in composition of the mucus and regulation of mucus secretion (Zalewsky and Moody, 1979). The cells are recognised as mucus secreting, by the mucin granules stored beneath the apical membrane of the epithelial cells and in the theca of the goblet cells. In most exocrine cells secretory granules are individually packaged and separated from each other by cytoplasm. In contrast mucin granules are tightly packed with membranes in close contact. Autoradiography of labelled mucins *in vitro* and *in vivo* have shown that mucus cells synthesize, transport and secrete mucin granules throughout their life-spans (Neutra and Leblond, 1966).

Three mechanisms of mucus secretion have been observed in a study on release of mucins by canine gastric mucosa. These are slow release by exocytosis, rapid release by apical expulsion and cell exfoliation (Zalewsky and Moody, 1979).

### 2.8.1. *Exocytosis*

Mucus secretion by slow exocytosis is the common method for macromolecular secretion, taking place in the pit region of the gland. The membranes of the mucin granules and the plasma interact, establishing continuity between the two

membranes (Zalewsky and Moody, 1979, Neutra and Forstner, 1987). A segment of the fused membranes is "pinched off" and mucus flows into the lumen. The lower segment of the mucin granule membrane, now continuous with the plasmalemma, remains to seal off the interior of the cell at the point of extrusion.

### 2.8.2. *Apical expulsion*

Apical expulsion is a rapid mucus secretory mechanism which involves the interfoveolar cells. The mucin granules swell within the cell with subsequent fusion of their membranes to form an intracellular mucus pool. The mucus streams into the lumen at an opening which has been left by "pinching off" of the plasma membrane (Zalewsky and Moody, 1979). Cells involved in this secretory mechanism show degenerative changes and the terminal event of secretion is *in situ* degeneration.

### 2.8.3. *Cell exfoliation*

Cell exfoliation is a rare form of mucus secretion. It involves the expulsion of the entire cell into the lumen. The cells are of older generations as they are generally foveolar cells with neutral staining mucins. This secretory mechanism together with apical expulsion rid the mucosa of senescent epithelial cells (Zalewsky and Moody, 1979). Exocytosis, on the contrary, is the mechanism of secretion of pit cells storing sulfated glycoproteins.

# CHAPTER 3.

# MATERIALS AND METHODS

# 3. MATERIALS AND METHODS

## 3.1. Chemicals and compounds

General laboratory reagents used for the extraction and isolation of mucins were obtained from Merck laboratories Darmstadt, West Germany. Phenylmethylsulphonyl fluoride (PMSF) *N*-ethylmaleimide (NEM) and ethylenediaminetetraacetic acid (EDTA), were also supplied by Merck laboratories, while caesium chloride (CsCl) was supplied by Boehringer Mannheim (SA) (Pty) Ltd.

Pararosanaline (C.I.42500), bovine serum albumin (BSA) and purified porcine mucin were obtained from Sigma Chemical Company St. Louis U.S.A. The Bio-Rad silver stain kit for protein was supplied by Bio-Rad Laboratories Ltd., Hertfordshire, U.K.

Acrylamide, *NN'*-methylenebisacrylamide, tetramethylethylenediamine and ammonium persulphate were obtained from British Drug Houses (BDH), Dorset, U.K. Calibration kits for molecular weight determination using electrophoresis were obtained from Pharmacia Fine Chemicals, Uppsala, Sweden.

Spectrapor dialysis membrane tubing with a cutoff point of 12,000 to 14,000 was ordered through Spectrum Medical Industries, Inc. Los Angeles, USA.

The gel filtration media (Sepharose CL-2B and Sepharose CL-4B, Sephadex G-50) used for chromatographic separation, were supplied by Pharmacia Ltd, Buckinghamshire, U.K.

A Hoefer SE600 slab gel system was used for polyacrylamide gel electrophoresis. Western blot analysis was kindly performed by Dr R Saunders in the Dept of Medicine, Liver Research Centre, Groote Schuur Hospital.

A Hitachi U 2000 spectrophotometer, a Hitachi Himac high speed centrifuge, a Beckman L8-70m ultracentrifuge and a Janke and Kunkel Ultra-Turrax were standard items of equipment used throughout the project. The equilibrium density gradients were formed in a Beckman 60 Ti titanium rotor using polyallomer tubes (capacity 38ml).

## 3.2. Collection of mucus

Mucus was obtained from 4 different groups for this study:

1. Human transplant donor stomachs (HD)
2. Human cadavers (PM)
3. Human gastric ulcer resection specimens (HGU) and
4. Human gastric cancer resection specimens (HCA).

Specimens from groups 1 (HD) and 2 (PM) were used as controls in this study. Transplant donor stomachs were obtainable within approximately 40 minutes of the patient being declared brain dead, whilst stomachs from cadavers were available only between 5 and 10 hrs after death. Total gastrectomy or antrectomy specimens were provided by surgeons within approximately 20 minutes of a patient having undergone surgery for peptic ulceration or carcinoma of the stomach. The diagnoses of conditions of the diseased stomachs are given in Tables 6 and 7. Factors such as age and sex of the individuals were not considered (Tables 4-7). The mucosae of these stomachs were scraped for mucus within 10-20 minutes of resection. Stomachs or resected specimens were opened along the greater curvature to expose the mucosal surface and the mucus was gently removed with a glass slide and collected in a container on ice. Samples were stored at -20°C until required.

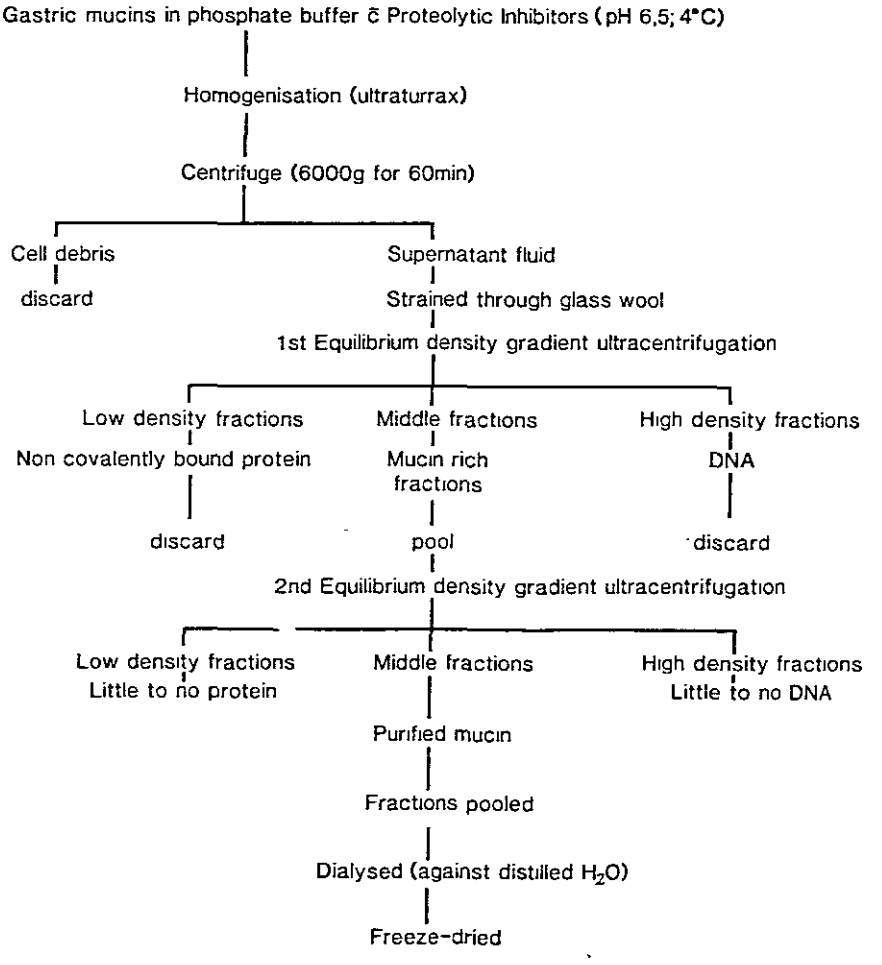
## 3.3. Solubilisation of mucus

The frozen crude mucus samples were thawed in 1mm PMSF (Mall et al, 1987) and then suspended in 4 volumes 15mM cold phosphate buffer containing 5mM EDTA and 5mM NEM, all at pH 6.5 (Carlstedt and Sheehan, 1984). After homogenisation with an Ultra-Turrax at maximum speed for 60 seconds the samples were spun at 6000g for 1h to remove insoluble debris (Allen, 1977). The supernatant was filtered through glass wool to remove excess lipid material. The samples were kept cold (4°C) throughout these procedures.

## 3.4. Isolation and purification of mucins

Mucins in the supernatant were isolated and purified by density gradient centrifugation twice in caesium chloride, at 105,000g for 48h (Creeth and Denborough, 1970, Starkey et al, 1974). The density of the supernatant was adjusted to 1.42g/ml by the addition of solid caesium chloride. After ultracentrifugation, tubes were divided into 9 equal fractions and the density of each fraction determined. The protein, glycoprotein and DNA content of each fraction was measured on an aliquot of each fraction after dialysis for 24 hours against distilled water to remove excess caesium chloride. The purified mucins obtained after ultracentrifugation (Starkey et al, 1974, Pearson et al, 1980) were pooled, dialysed against distilled water and freeze dried.

Figure 8 outlines the steps followed for the solubilisation and purification of the gastric mucus glycoproteins.



## 3.5. Analytical procedures

### 3.5.1. *Glycoprotein estimation*

In this study mucins were detected by the periodic acid-Schiff (PAS) reaction, a widely used staining technique for carbohydrates. Other methods used for the determination of glycoprotein, include the anthrone or orcinol procedures but these have proved to be less sensitive and prone to interference from free protein and nucleic acids. Measurement at 280nm would also be inaccurate due to the presence of free protein and the lack of aromatic acids.

The chemical basis of the PAS reaction is that adjacent 1:2 glycol or 1:2 amino-hydroxy groups of individual monosaccharides, are oxidised by periodic acid and converted into aldehydes; these aldehydes are reactive to Schiff reagent, producing a pink colour. In this study the Schiff reagent was prepared by dissolving 1gm pararosaniline (Sigma, Schiff base) in 100ml boiling water, cooling the solution to 50°C and then addding 20ml 1M HCl. This solution was twice mixed with 300mg activated charcoal, shaken for at least 5 mins and filtered through Whatman No.1 filter paper. The resultant deep red solution was stored in an amber glass bottle at room-temperature. Before use, 60 ml of this stock solution was incubated at 37°C with 1gm of sodium-metabisulphite for 60 mins. The decolourised solution was used for the assays and for staining of the polyacrylamide

gels. Aliquots of each fraction after caesium chloride density gradient ultracentrifugation were dialysed against distilled water to remove excess caesium chloride thus preventing non-specific interference in the PAS reaction. Eluted fractions after column chromatography did not present this problem and could be assayed with the Schiff reagent directly. Samples were incubated with periodic acid for 60 mins at 37°C. Decolourised Schiff reagent was added to the oxidised samples and after 30mins the sample absorbances were read at 555nm.

Standard curves (20-180mcg in 0.2M sodium chloride and 0.02% (w/v) sodium azide) were constructed from pig gastric mucin which had been purified twice in caesium chloride (see Appendix A).

3.5.2. *Protein estimation*

The protein content of density gradient and gel filtration fractions was estimated by the method of Lowry (1951) which is a combination of the Biuret and Folin-Ciocalteu methods. It is widely used in research to measure tissue and enzyme proteins, detecting proteins in concentrations as low as 10ug/ml. The Biuret method depends on the presence of peptide bonds present in all proteins and as such measures total protein in a given sample. The Folin-Ciocalteu method depends on the reduction of phosphotungstic-phosphomolybdic acid by tryptophan and tyrosine present in most proteins to give a blue colour. An alkaline copper solution is added to appropriately diluted samples. After standing at room temperature for at least 10 mins diluted Folin-Ciocalteu reagent is added and the sample is immediately mixed. The colour is allowed to develop for 30-40 mins and sample absorbances are read at 700nm.

Varying concentrations (20-100mcg) of bovine serum albumin in 0.2M sodium chloride and 0.02% (w/v) sodium azide were used to construct a standard curve (see Appendix B).

### 3.5.3. *DNA estimation*

Deoxyribonucleic acid content was estimated by measuring the absorbances of samples, diluted in distilled water, at 260nm. The method is suitable for DNA estimation in samples not significantly contaminated with protein or other nucleic acids. At 260nm an OD of 1 corresponds approximately to 50mcg/ml for double stranded DNA.

## 3.6. Biochemical procedures

### 3.6.1. *Dialysis*

Samples were dialysed exhaustively at 4°C for a minimum of 48hrs, against at least four changes of distilled water. Spectrapore dialysis membrane tubing with a molecular weight cutoff of 12,000 to 14,000 was rinsed with distilled water and then boiled in an aqeous solution of 0.5% sodium bicarbonate/EDTA for 1 minute. Samples were dialysed, with continuous stirring, against volumes ~ 100fold the volume of the sample. Large glass beakers (10L capacity) were used for this purpose.

### 3.6.2. *Freeze drying*

Dialysed mucin samples were transferred to Virtis vacuum flasks which were immersed in liquid nitrogen and by gentle rotation of the flasks, samples were frozen. The Virtis vacuum flasks were connected to a Christ (Alpha 1-5) freeze drier and samples were dried at -54°C at a rate of 5kg/24hrs. Lyophilised mucins were stored at -20°C until required.

### 3.6.3. *Column Chromatography*

Gel filtration chromatography is used for the determination of the molecular weight and the purification of mucins (Forstner et al, 1973). In order to establish the ratio of polymeric to degraded mucin in this study, Sepharose CL-2B and Sepharose CL-4B gel filtration media was used. Sepharose CL is a cross-linked bead formed gel prepared from agarose and is suitable for use with eluents containing a high salt concentration and is usable in the pH range 3-14.

All columns were prepared as described in the Pharmacia handbook "Gel filtration - theory and practice" (obtained from suppliers). Sepharose 2B and 4B and Sephadex G-50 were wetted in 0.2M sodium chloride/0.02% (w/v) sodium azide and degassed (using a Venturi pump) before being poured at room temperature. Columns (1.6cmx100cm) were calibrated with 0.1% (w/v) dextran blue solution containing 0.05% (w/v) methyl orange and equilibrated with 0.2M sodium chloride/0.02% (w/v) sodium azide for at least 36 hours before use. The total bed volumes ranged between 195ml and 205ml. Freeze dried samples were reconstituted in 0.2M NaCl:0.02% (w/v) sodium azide and loaded onto the top of the column. All columns were eluted by downward flow (20mls/hr) with 0.2M NaCl:0.02% sodium azide with the aid of a Gilson peristaltic pump. Eluted fractions (2ml for Seph 2B and 4B; 1ml for Sephadex G-50) were collected by an L.K.B. Ultrorac fraction collector and assayed for their protein and mucin content.

3.6.4. *Polyacrylamide gel electrophoresis*

Polyacrylamide gel electrophoresis (Laemmli 1970), using 7.5% gels or 4-20% gradient gels, was employed to detect the purity and the extent of degradation of the mucins. Up to 850mcg mucin (~100ul) was loaded onto gels stained for carbohydrate by the PAS method (Dubray and Bezard, 1982). Between 150 and 250mcg (~25ul) was loaded when gels were stained for protein with $AgNO_3$ (Bio-Rad kit). Between 100 and 150 microlitres of each density gradient fraction were electrophoresed and stained for protein with Coomassie blue or $AgNO_3$.

All gels were prepared from a stock solution of 30% acrylamide/0.8% *N,N*-methylenebisacrylamide (w/v). Running gels were of either 7.5% or 4%-20% acrylamide concentration, 0.375M Tris-HCl (pH 8.8), 0.1% SDS and up to 8% glycerol. Stacking gels contained 3% acrylamide, 0.125M Tris-HCl (pH 6.8) and 0.1% SDS. Freeze-dried samples were reconstituted in sample application buffer (0.0625M Tris, 2% SDS, 10% glycerol) pH 6.8, with or without 0.2M 2-mercaptoethanol. Bromophenol blue was used as a tracking dye. Proteins were denatured by boiling for 2 minutes and when possible samples were allowed to stand overnight to ensure complete reduction. The reservoir buffer (pH 8.8) contained 0.025M Tris-HCl, 0.192M glycine and 0.1% SDS (w/v). Electrophoresis was carried out in the cold (4°C) at a constant current of 40mAmps per gel until the tracking dye reached the bottom of

the gel (6-7 hours). After electrophoresis, gels were immersed in the appropriate solutions in Pyrex trays and gently agitated on a shaker. Surface evaporation was prevented by covering the trays with plastic sheets. Samples and molecular weight markers were electrophoresed simultaneously; when gels were to be stained for mucin, the gel was cut between the first sample and the molecular weight markers making it possible for the latter to be stained for protein.

On staining for mucin the gels were fixed in 25% isopropyl alcohol/10% acetic acid overnight at room temperature. This solution was discarded after ~16 hours and replaced with 7.5% acetic acid for 30 mins. Gels were further immersed in 0.2% aqueous periodic acid and stored at 4°C for 60 mins. The periodic acid was replaced with decolourised Schiff's reagent and stored at 4°C for 60 mins. Thereafter gels were decolourised with several changes of aqeous 7.5% acetic acid until the bands were clear. When stained for protein, gels were fixed and stained with 0.2% Coomassie Brilliant Blue in 50% ethanol/10% acetic acid overnight at room temperature. Gels were destained with frequent changes of a 25% ethanol/10% acetic acid solution, until the background was clear and the protein bands were clearly visible. On staining gels for protein with the Bio-Rad $AgNO_3$ kit, instructions (according to bulletin 1089 "The Bio-Rad silver stain") for 1mm thick polyacrylamide gels were strictly adhered to.

# CHAPTER 4.

# RESULTS

# 4. RESULTS

## 4.1. Solubilisation and purification of mucins

Crude mucus scrapings in a mixture of proteinase inhibitors at pH 6.5 were homogenised briefly at 4°C (60 secs) and the insoluble cell debris removed by centrifugation. The lipid in the supernatant was removed by filtering the material through glass wool. The mucins in the supernatant were then separated and isolated from contaminant protein, lipid and nucleic acids by equilibrium centrifugation in a caesium chloride density gradient under the conditions described in 3.4 (Starkey et al, 1974, Pearson et al, 1980).

After ultracentrifugation each tube was divided into 9 equal fractions and the density of each recorded; each fraction was then analysed for its mucin, protein and DNA content. Aliquots of each fraction were also subjected to SDS-PAGE to monitor the removal of protein and the successful purification of the mucin.

Successful gradients were accomplished for material from all groups as shown by the measurement of the density of each fraction (Figs. 9 and 10 a-d; representative for each of the 4 preparations: HD, PM, HGU and HCA). Density values for each sample ranged from ~1.27g/ml at the top of the tube to ~1.56g/ml at the bottom of the tube. After the first fractionation in a caesium chloride gradient, protein was

found in all fractions, the amounts decreasing with increasing density (Fig. 9 a-d). Since a considerable amount of protein was still detectable in the mucin rich fractions after the first ultracentrifugation step in caesium chloride, the mucin rich fractions were pooled and prepared for a second density gradient centrifugation, after which mucins were shown to be free of non-covalently bound protein and DNA (Fig. 10 a-d). In either density gradient mucin was mainly found in fractions 4-7 between a density range of 1.35 and 1.45 g/ml (mean of 1.41g/ml). In the PM group fraction 9 often showed a positive reaction with PAS (Fig. 11 a and b). Most of the DNA content of the samples fractionated at a density of ~1.56g/ml *i.e.* fraction 9.

The minimal amount of protein detected after the second purification step is seen in either the low density fractions (fractions 1 and 2) (Fig. 10 d), or in the mucin rich fractions (Figs. 10 a, 10 c).

Table 1 shows the mucin, protein and DNA content expressed as a percentage of the total material per sample after each spin, for all groups.

In the HD group, the amount of protein (46,39% of the total material) and mucin (44,76% of the total material) were similar after the first density gradient. Protein levels however, decreased to 14,97% while the mucin content increased to 74,67% after the second equilibrium density

gradient ultracentrifugation step (see Table 1). The DNA content increased from 8,77% of the total material after the first purification step to 10,47% of the total material after the second density gradient.

The PM samples behaved anomalously in that the protein content (19,11% of the total material) was lower than the mucin content (66,31% of the total material) after the first equilibrium density gradient ultracentrifugation. Table 1 shows that the amount of protein decreased even further (5,64%) after a second equilibrium density gradient ultracentrifugation, with mucin accounting for 85,08% of the total material. The amount of DNA present in the PM sample decreased from 14.6% of the total after the first density gradient to 9,32% of the total material after the second density gradient in caesium chloride.

Samples from the HGU group showed a vast difference between the protein content (66,84% of the total material) and the mucin content (26% of the total material) at the end of the first equilibrium density gradient ultracentrifugation, a situation which was reversed after the second density gradient i.e 80,38% mucin and 13,12% protein (Table 1). The DNA content differed slightly between the first (7,23% of the total) and the second (6,81% of the total) purification steps.

Samples of HCA mucin behaved in a similar manner to the HGU samples i.e. 24,61% mucin and 68,46% protein at the end of the first density gradient ultracentrifugation in caesium chloride. These results were reversed with the second density gradient i.e. the mucin content increased to 76,11% while the protein value decreased to 14,95%. The DNA content increased from 6,93% of the total material to 9,07% of the total material (Table 1) between the first and second purification steps.

Figure 12 shows the relationship between the mucin, protein and DNA content expressed as the total amount of each component measured after each ultracentrifugation step. PM mucus yielded the most mucin after purification when compared to the mucin content of other groups. PM stomachs yielded a larger amount of scrapable mucus on the mucosal surface which in turn could have led to the higher yield of purified mucins. The large amount of contaminant protein seen in the HCA samples after the first purification was reduced to a minimum after the second equilibrium density gradient ultracentrifugation (Fig. 12).

SDS-PAGE analysis of fractions obtained after each of three ultracentrifugation steps for the purification of mucins from a HCA mucus sample, is shown in Plates 1 a, b, and c. The gels were stained for protein with $AgNO_3$ (Bio-Rad kit) or Coomassie Blue. The progressive decrease in protein content (stained with BCB) from fraction 1 to fraction 9 shown after

the first purification step (Plate 1a, lanes 2-9) is clearly evident. Plate 1b (stained with $AgNO_3$) shows the gradient fractions after a second caesium chloride equilibrium density gradient ultracentrifugation. A fair amount of low molecular weight bands ($M_w$~58kDa), possibly due to non-covalently bound protein, can be seen for fractions 1-6 in lanes 1-6 (Plate 1b). Large molecular weight mucins staining prominently in the stacking gel and at the top of the running gel are seen in lanes 4-7 (Plate 1b). Due to the comparatively high protein content, this particular sample required a third purification step (Plate 1c). Smears of glycoprotein material (stained with $AgNO_3$) can be seen in the stacking and running gels (Plate 1c lanes 4-8) with the protein ($M_w$~67kDa) in lanes 1-4 decreasing in intensity and disappearing completely in lanes 5-9. If the fractions 4-8 were pooled and considered to be pure mucin, the presence of a protein band at $M_w$67kDa in lane 4 (fraction 4) would account for less than 0.01% of the total mucin after the pooling of fractions 4-8.

4.1.1. SUMMARY

If we are to understand how mucus protects the gastric mucosa against acid and pepsin, it is necessary to isolate and characterise the component mucins in as near a native state as possible, while still retaining the viscous and gel forming properties of the secretion (Allen et al, 1989).

Modification of the mucins through proteolytic degradation is a distinct possibility unless precautions are taken to inhibit endogenous proteinases. In the present study human gastric mucus gel was solubilised by high-speed homogenisation in cold phosphate buffer at pH 6,5 supplemented with proteolytic inhibitors. Phenylmethylsulphonyl fluoride and EDTA were used to inhibit serine and metalloproteinases respectively. *N*-ethylmaleimide inhibits thiol proteinases and limits possible thiol-disulphide exchange, whilst pepsin would be inactive at pH 6,5. Carlstedt and Sheehan, (1984) are of the opinion that high-speed homogenisation causes mechanical degradation of mucins; however according to Robson et al, (1975) and Mall, (1988), gastric mucins are not degraded by this method of extraction. Mucins were separated from contaminant protein and DNA by equilibrium density gradient ultracentrifugation in CsCl (Creeth and Denborough, 1970, Starkey et al, 1974). This method separates the mucin on the basis of its density from the lower density protein and the higher density nucleic acids and at the same time, the high ionic strength of the

caesium salt (3.5M CsCl) counteracts the strong non-covalent interactions between macromolecules e.g. protein-glycoprotein interactions (Allen, 1981). An added advantage of equilibrium density gradient ultracentrifugation is the concentration of mucin molecules rather than dilution which may be the case with an alternative purification method such as gel filtration chromatography (Carlstedt and Sheehan, 1984). The criteria whereby purified mucin was declared free of contaminant protein were: (1) no protein in the low density fractions after a second caesium chloride density gradient (2) the lack of low molecular weight bands on a polyacrylamide gel stained for protein (Forstner et al, 1973) and (3) the absence of DNA as evidenced by U.V. spectroscopy. The risk of degradation was minimised by the extraction of mucins in the cold (4°C) and the use of proteolytic inhibitors at pH 6.5 which inhibits the action of pepsin (Carlstedt and Sheehan, 1984, Allen et al, 1984). The high protein content present at the end of the first fractionation is most likely due to cellular debris, haemoglobin, lipoprotein and serum glycoproteins which are non-covalently bound to the mucins (Creeth, 1978). In the case of the diseased stomachs the high protein content could be attributed to haemoglobin as these mucus scrapings were variably bloodstained. The quite high protein content of HCA specimens could be due to the excessive amount of serum albumin that passes into the lumen during the disease (Creeth, 1978). HD mucus scrapings were often contaminated with food particles and perhaps this artificially increased

the protein value. This protein is located in the top 2-3 fractions of the gradient which are discarded once the mucin rich fractions are identified. The minor amount of protein seen within the area of the mucin peak after the second purification step very likely is covalently bound protein, an integral part of the mucin. Fractions between densities 1.35 and 1.45 gm/ml, over fractions 4-7 were shown to contain most of the mucin. In some instances fraction 9 of the PM group showed a positive reaction with PAS. However due to the interval between death and scrape, autolysis could have occurred, releasing proteoglycans which react positively with PAS.

After two fractionations in a caesium chloride gradient samples from all groups were shown to be free of contaminant protein and DNA (Fig. 10 a-d). Fig. 12 shows mucin was present in amounts greater than that for protein and DNA in the HD, HGU and HCA groups after the second density gradient only. In the PM group mucin was present as the largest component even after the first fractionation and overall the PM group yielded the most purified mucin (Table 1). The high yield of mucins in the PM group was not unexpected considering the increased volume of scrapable mucus on the mucosal surface of cadaver stomachs. In a separate study it has been shown that pig stomachs left *in situ* after sacrifice at intervals for up to 18 hours and stored at 4°C gave increased volumes of scrapable mucus with time (Mall personal communication). However the ratio of degraded to polymeric

mucin was greater in this group. Post mortem mucus from humans and animals are used as normal controls by researchers but its suitability is questionable in the light of these observations. The established method of purification of mucins, *viz* density gradient centrifugation in caesium chloride, used in laboratories all over the world was found also to be adequate for the purposes of this study.

**Table 1**

| mat. Group | n | CsC1 Stage | Composition (% by weight of tot.mat.) Mucin | Protein | DNA | Tot. mg/ml |
|---|---|---|---|---|---|---|
| Human Donor | 4 | 1st | 44.8 | 46.4 | 8.8 | 14.9 |
| | | 2nd | 74.7 | 14.9 | 10.5 | 4.3 |
| Post Mortem | 5 | 1st | 66.3 | 19.1 | 14.6 | 32.0 |
| | | 2nd | 85.1 | 5.6 | 9.3 | 10.4 |
| Human Gastric Ulcer | 12 | 1st | 26.1 | 66.8 | 7.2 | 11.2 |
| | | 2nd | 80.4 | 13.1 | 6.8 | 2.0 |
| Human Gastric Cancer | 13 | 1st | 24.6 | 68.5 | 6.9 | 23.0 |
| | | 2nd | 76.1 | 15.0 | 9.1 | 2.6 |

Protein and DNA content of purified gastric mucins of all groups. Mucins were purified and isolated by equilibrium density gradient ultracentrifugation. Mucin, protein and DNA content were determined and expressed as a percentage of the total material.

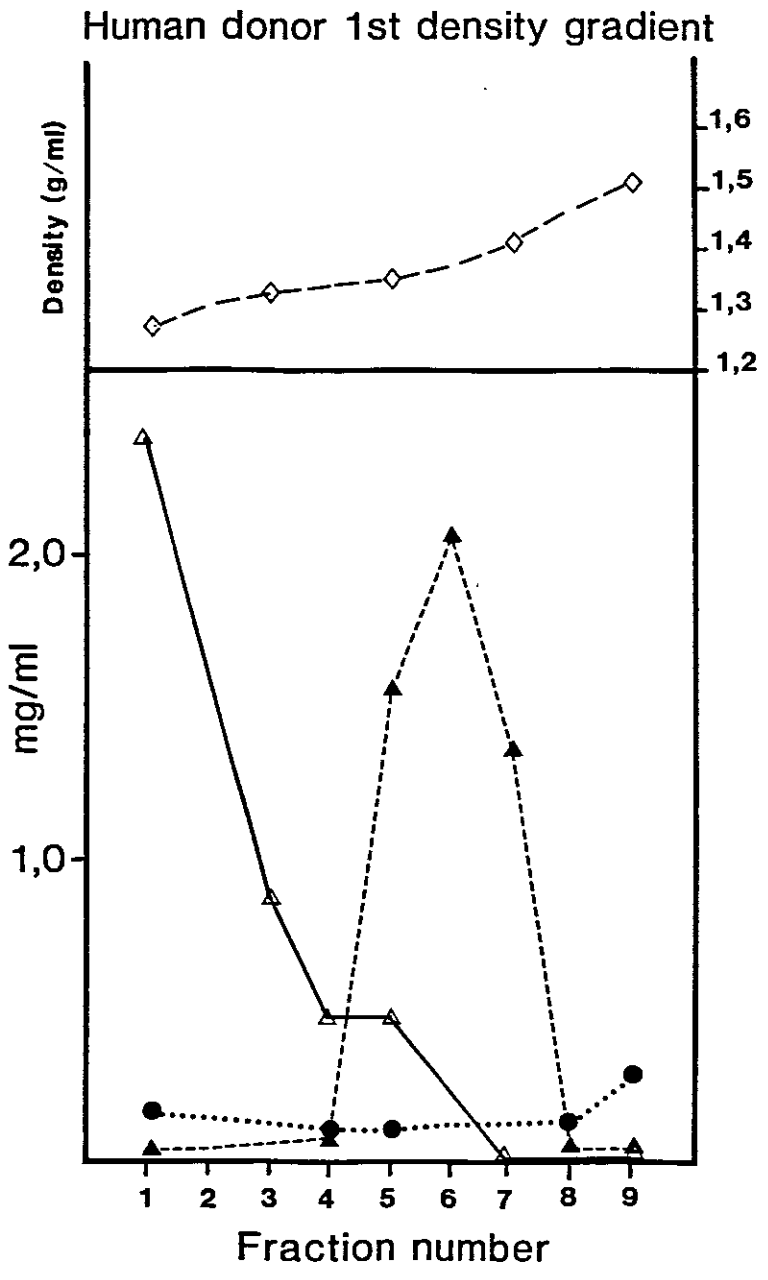



Figure 9a

The starting density of the caesium chloride was 1.42 g/ml and the resultant gradient was fractionated into 9 equal parts. The density of each fraction was measured (◇--◇) and following exhaustive dialysis against distilled water, each fraction was analysed for glycoprotein with PAS (▲-▲), protein with Lowry (△—△) and DNA by absorbance at $A_{280}$(●··●).

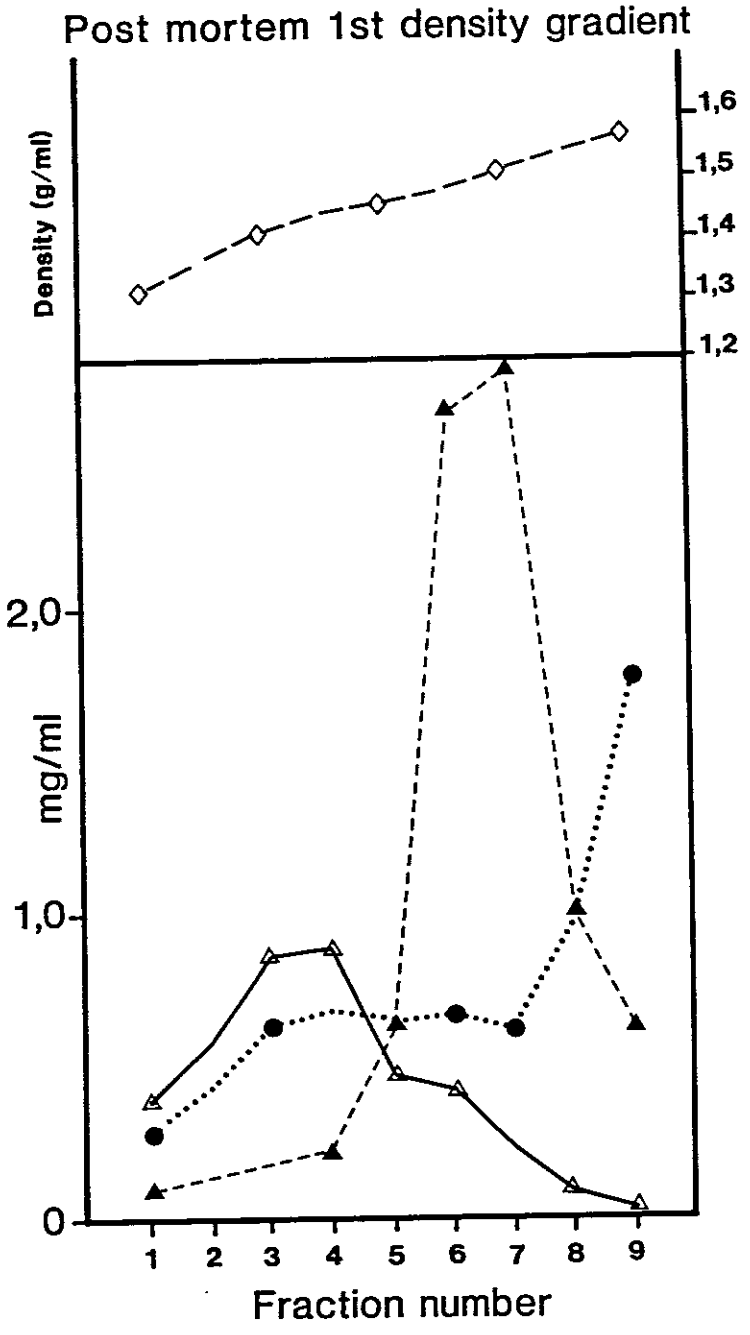



Figure 9b

The starting density of the caesium chloride was 1.42 g/ml and the resultant gradient was fractionated into 9 equal parts. The density of each fraction was measured (◇--◇) and following exhaustive dialysis against distilled water, each fraction was analysed for glycoprotein with PAS (▲-▲), protein with Lowry (△—△) and DNA by absorbance at $A_{280}$ (●·●).

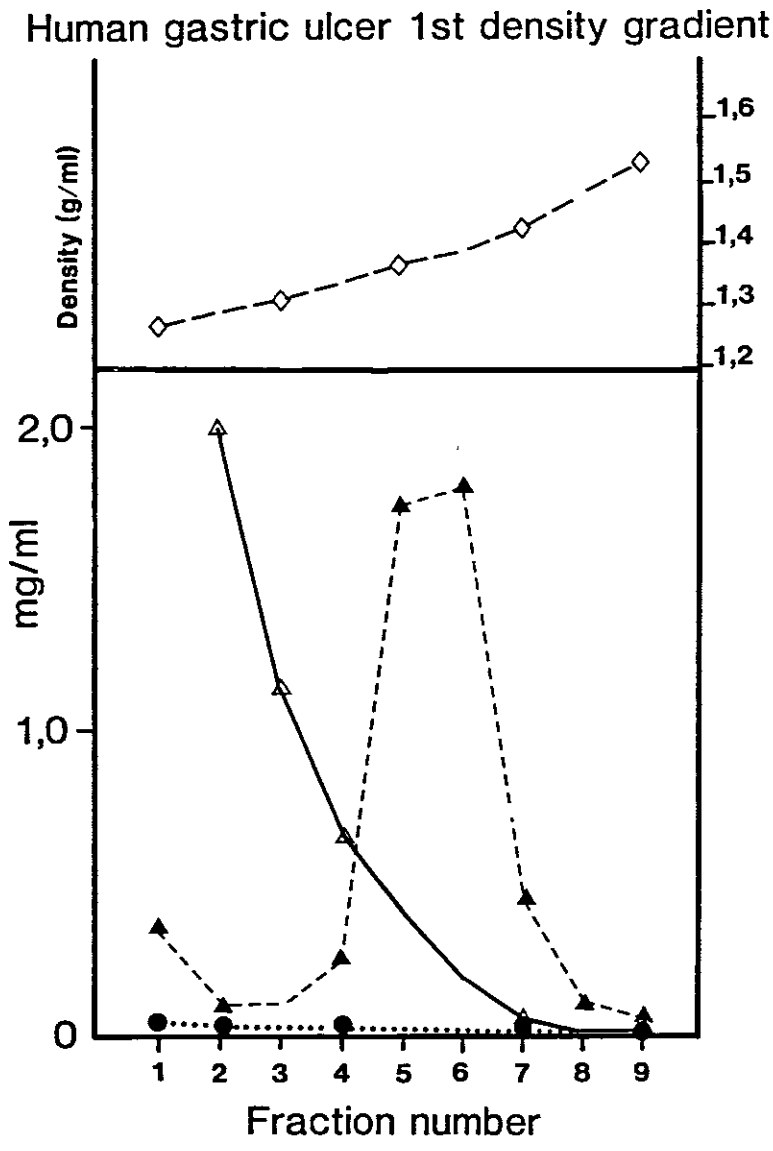



Figure 9c

The starting density of the caesium chloride was 1.42 g/ml and the resultant gradient was fractionated into 9 equal parts. The density of each fraction was measured (◇--◇) and following exhaustive dialysis against distilled water, each fraction was analysed for glycoprotein with PAS (▲-▲), protein with Lowry (△—△) and DNA by absorbance at $A_{280}$(●··●).

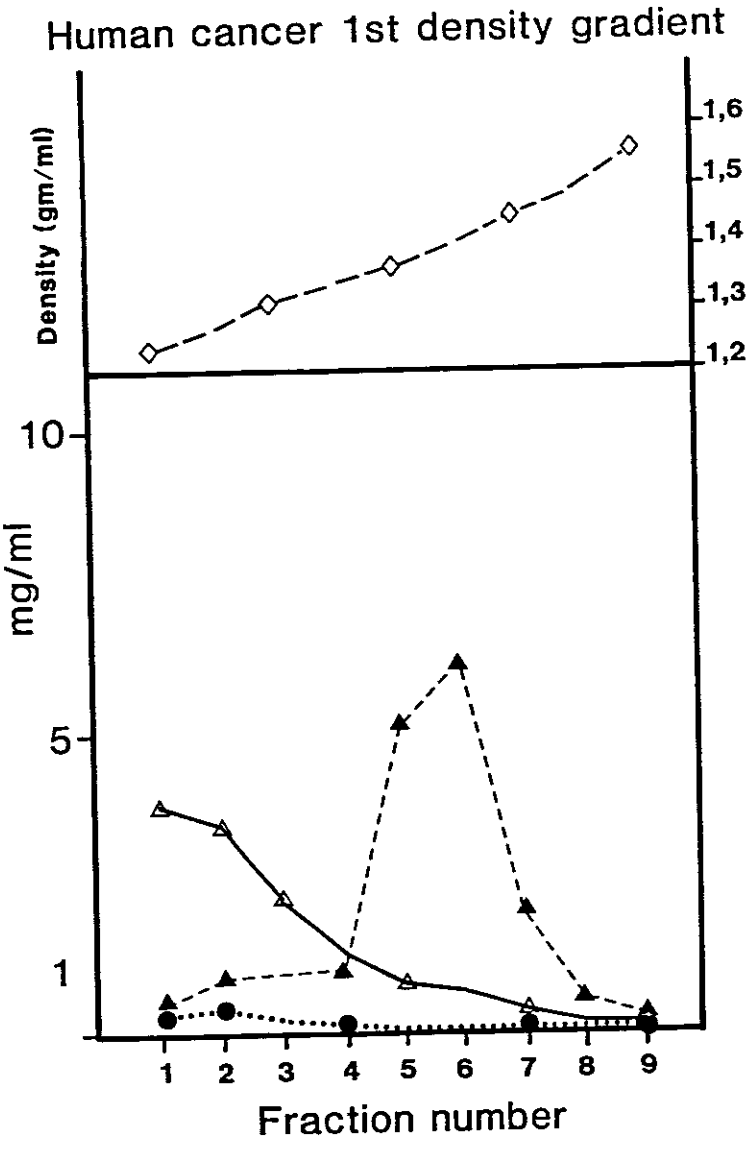



Figure 9d

The starting density of the caesium chloride was 1.42 g/ml and the resultant gradient was fractionated into 9 equal parts. The density of each fraction was measured (◇--◇) and following exhaustive dialysis against distilled water, each fraction was analysed for glycoprotein with PAS (▲--▲), protein with Lowry (△—△) and DNA by absorbance at $A_{280}$ (●··●).

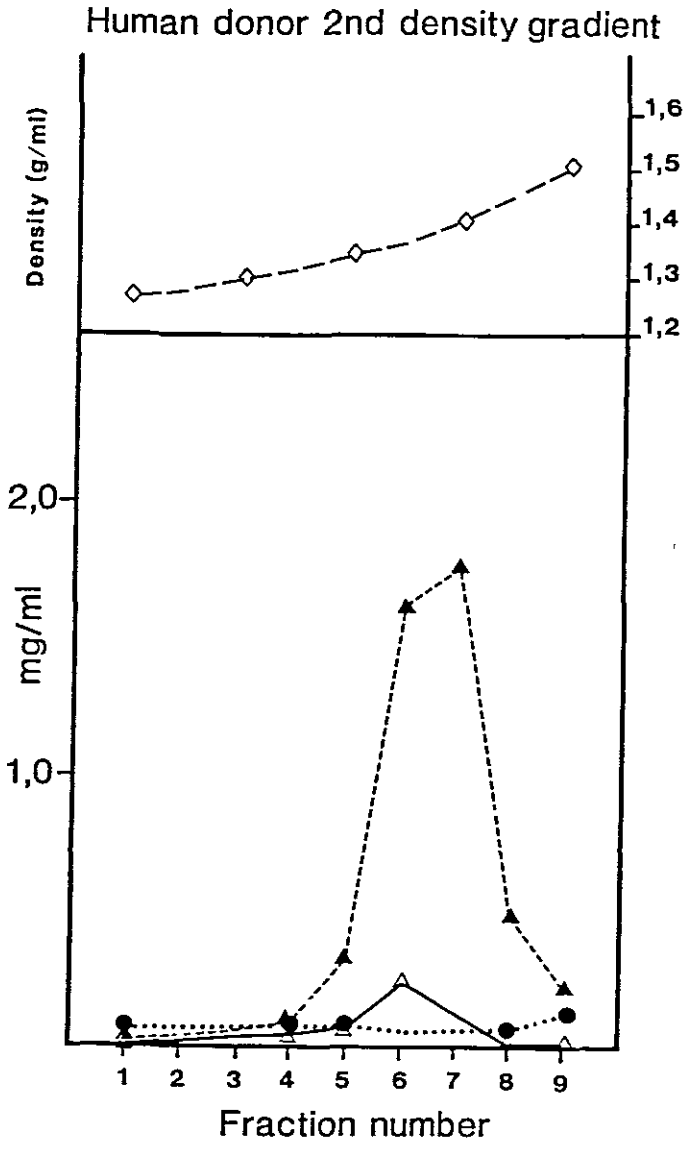



Figure 10a

Pooled mucin fractions from a previous fractionation in a CsCl density gradient were adjusted to a density of 1.42 g/ml and centrifuged at 105,000g for 48 hours at 10°C. The resultant gradient was fractionated into 9 equal parts. The density of each fraction was measured (◇--◇) and following dialysis against distilled water each fraction was analysed for glycoprotein (▲--▲), protein (△—△) and DNA (●··●).

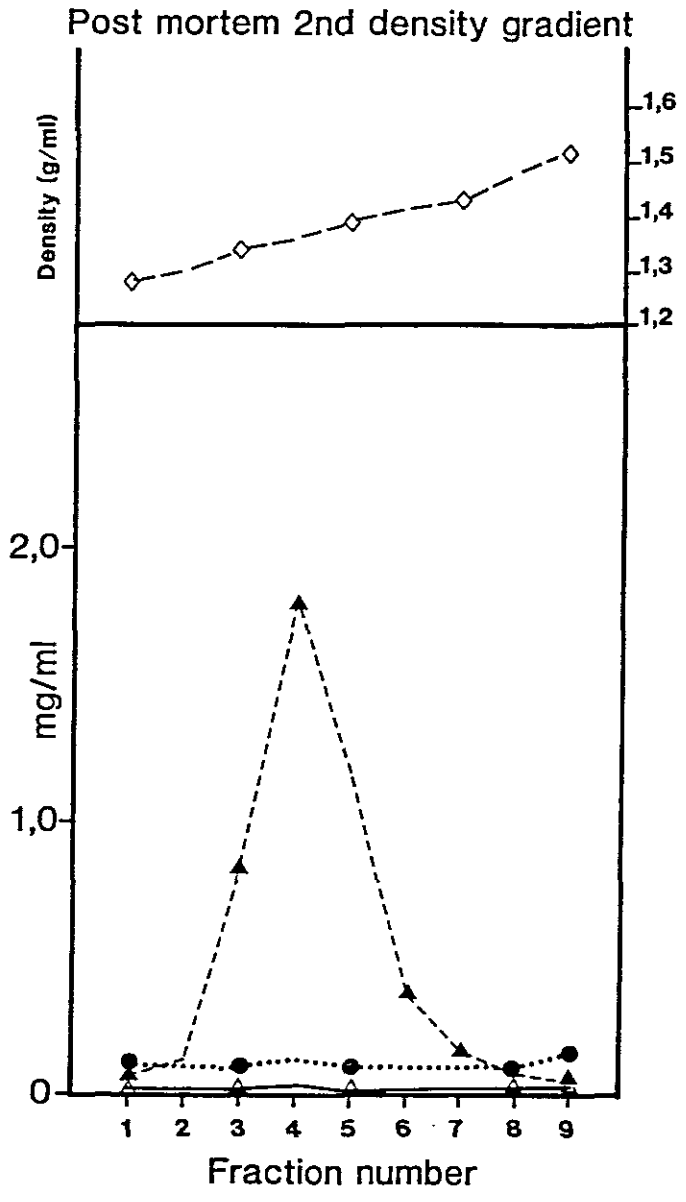



Figure 10b

Pooled mucin fractions from a previous fractionation in a CsCl density gradient were adjusted to a density of 1.42 g/ml and centrifuged at 105,000g for 48 hours at 10°C. The resultant gradient was fractionated into 9 equal parts. The density of each fraction was measured (◇--◇) and following dialysis against distilled water each fraction was analysed for glycoprotein (▲-▲), protein (△—△) and DNA (●··●).

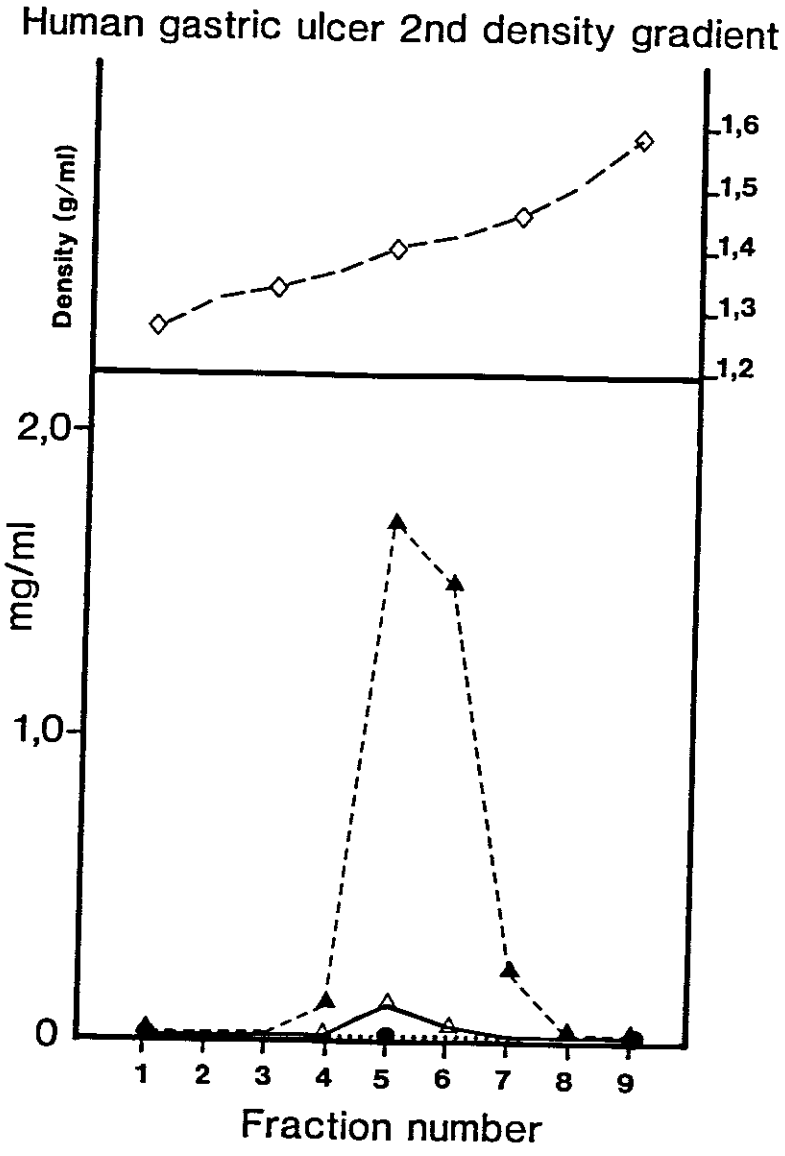



Figure 10c

Pooled mucin fractions from a previous fractionation in a CsCl density gradient were adjusted to a density of 1.42 g/ml and centrifuged at 105,000g for 48 hours at 10°C. The resultant gradient was fractionated into 9 equal parts. The density of each fraction was measured (◇--◇) and following dialysis against distilled water each fraction was analysed for glycoprotein (▲--▲), protein (△—△) and DNA (●··●).

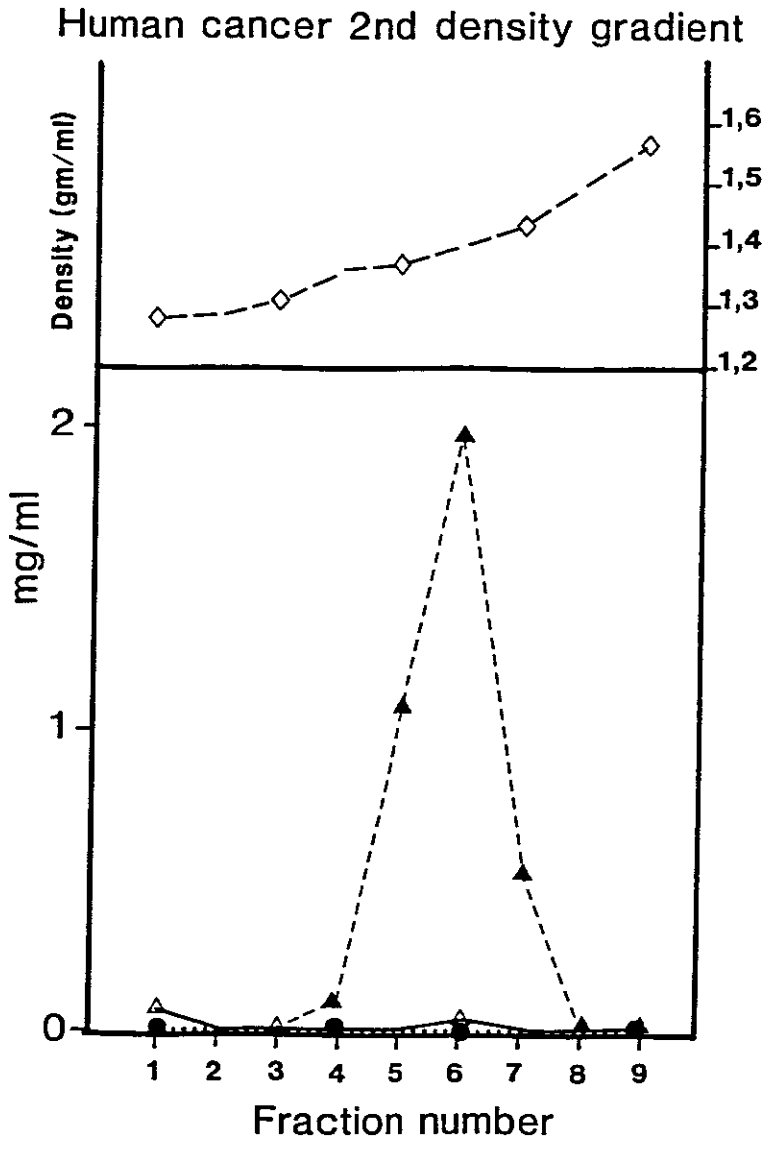



Figure 10d

Pooled mucin fractions from a previous fractionation in a CsCl density gradient were adjusted to a density of 1.42 g/ml and centrifuged at 105,000g for 48 hours at 10°C. The resultant gradient was fractionated into 9 equal parts. The density of each fraction was measured (◇--◇) and following dialysis against distilled water each fraction was analysed for glycoprotein (▲--▲), protein (△—△) and DNA (●··●).

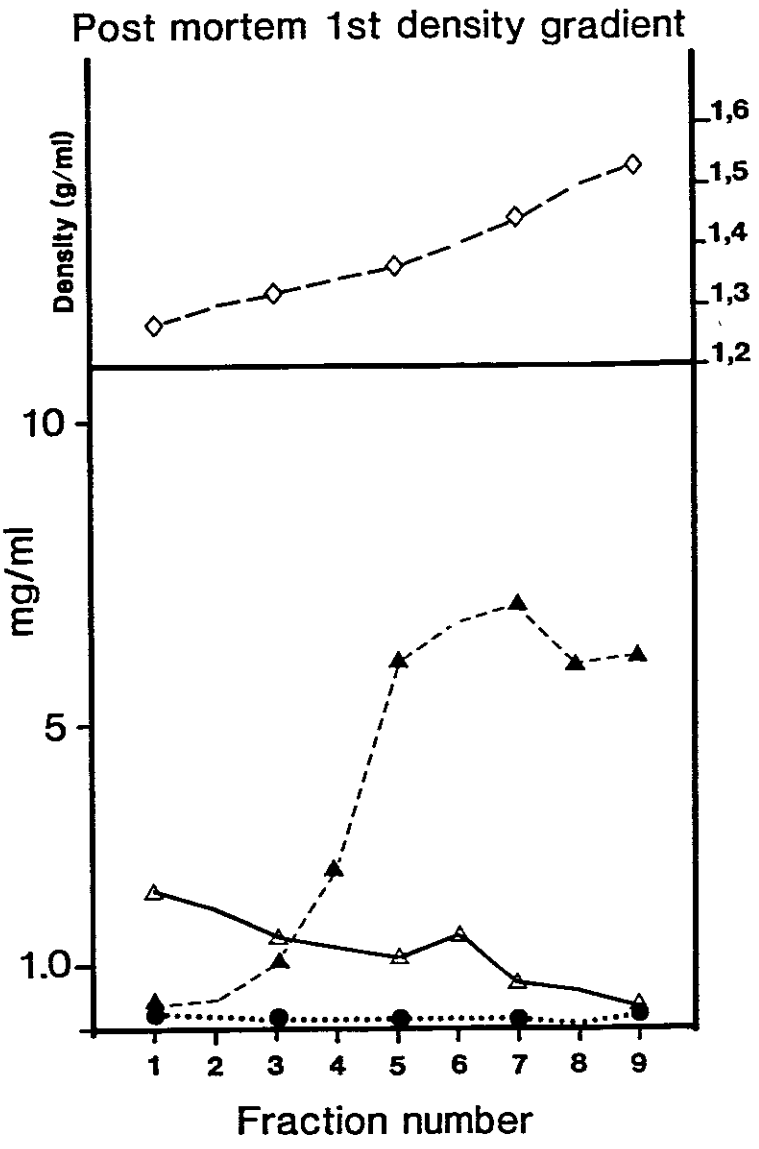



Figure 11a

Fractionation of post mortem mucus by density gradient centrifugation in a CsCl gradient. The gradient was divided into 9 equal fractions. The density (◇--◇) of each fraction was measured. After exhaustive dialysis against distilled water, each fraction was analysed for glycoprotein (▲--▲), protein (△—△) and DNA (●··●).

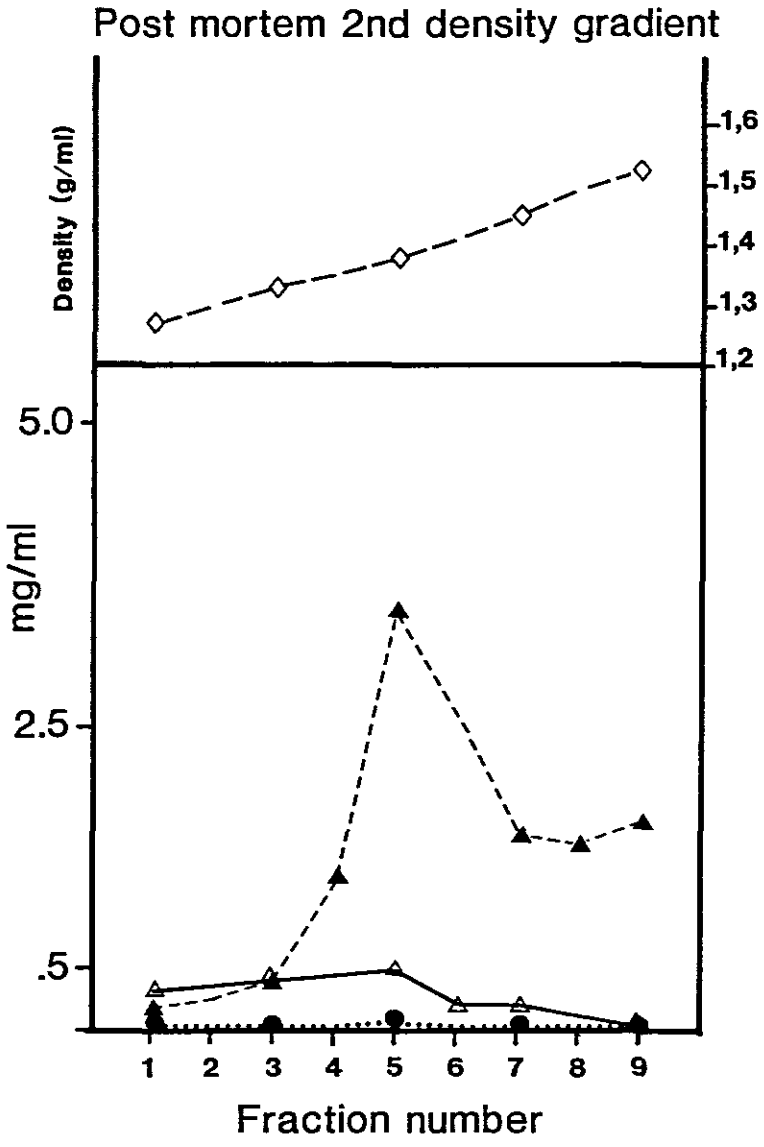



Figure 11b

Fractionation of post mortem mucus by density gradient centrifugation in a CsCl gradient. The gradient was divided into 9 equal fractions. The density (◇--◇) of each fraction was measured. After exhaustive dialysis against distilled water, each fraction was analysed for glycoprotein (▲--▲), protein (△—△) and DNA (●··●).

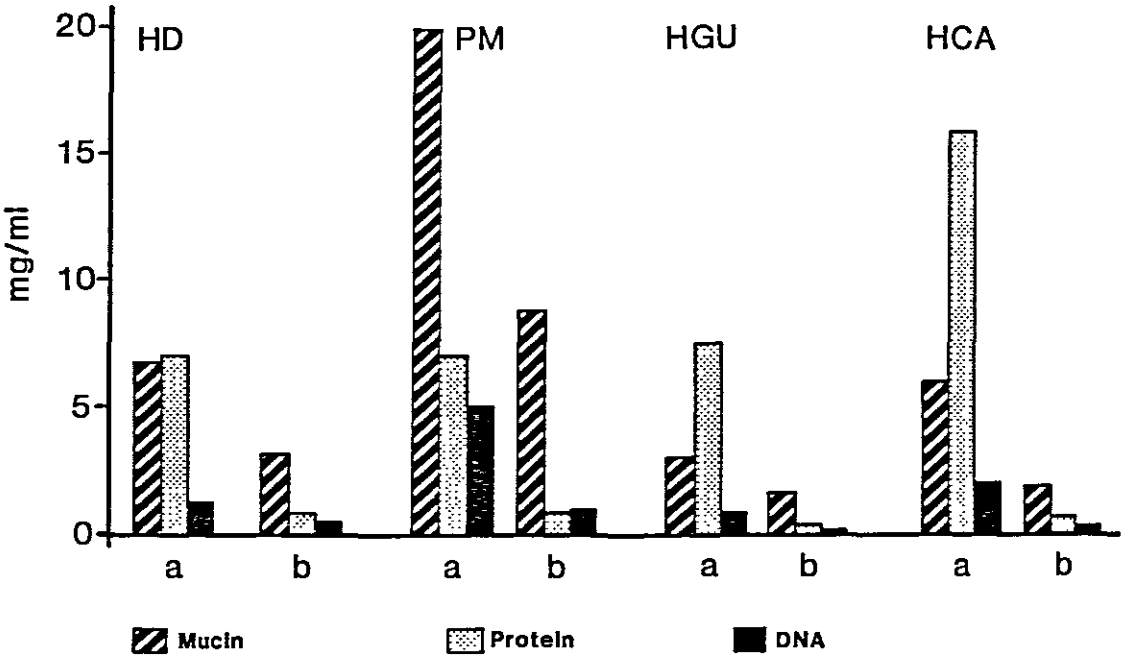



Figure 12

Total amount of mucin, protein and nucleic acid (mcg/ml) after the first (a) and second (b) equilibrium density gradient ultracentrifugation in caesium chloride.

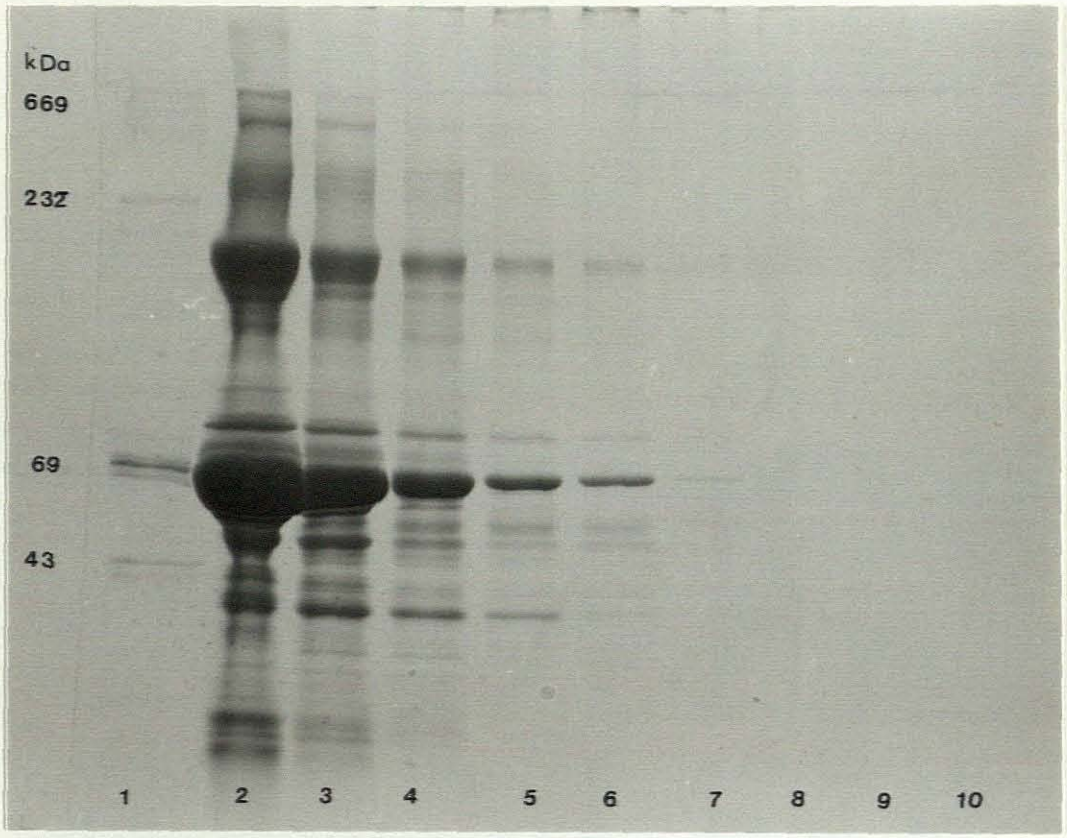



Plate 1a

Equilibrium density gradient ultracentrifugation fractions were electrophoresed on a 4-20% polyacrylamide gradient gel and stained for protein with Brilliant Coomassie Blue.

Lane 1 : High molecular weight marker.

Lanes 2-10: Fractions 1-9.

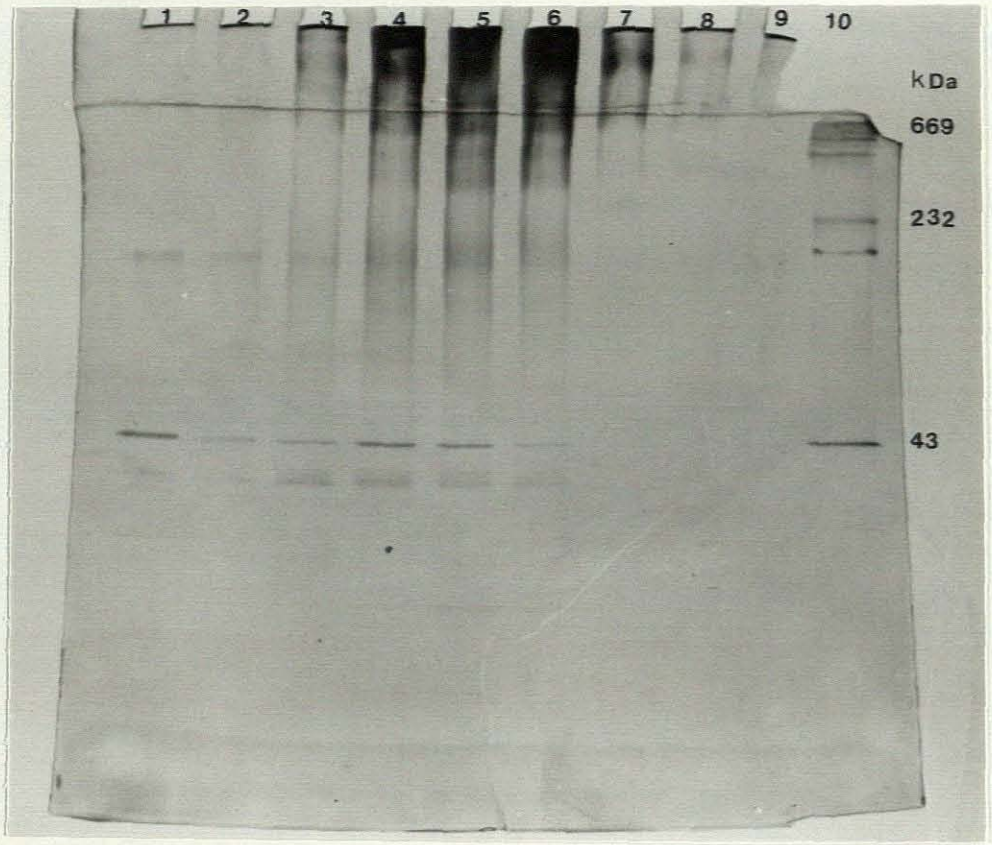



Plate 1b

Mucin rich fractions obtained after the first equilibrium density gradient ultracentrifugation were pooled and subjected to a second equilibrium density gradient in caesium chloride. The resultant fractions were electrophoresed on a 4-20% polyacrylamide gradient gel and stained for protein with the Biorad silver stain kit.

Lanes 1-9: Fractions 1-9.

Lane 10: High molecular weight marker.

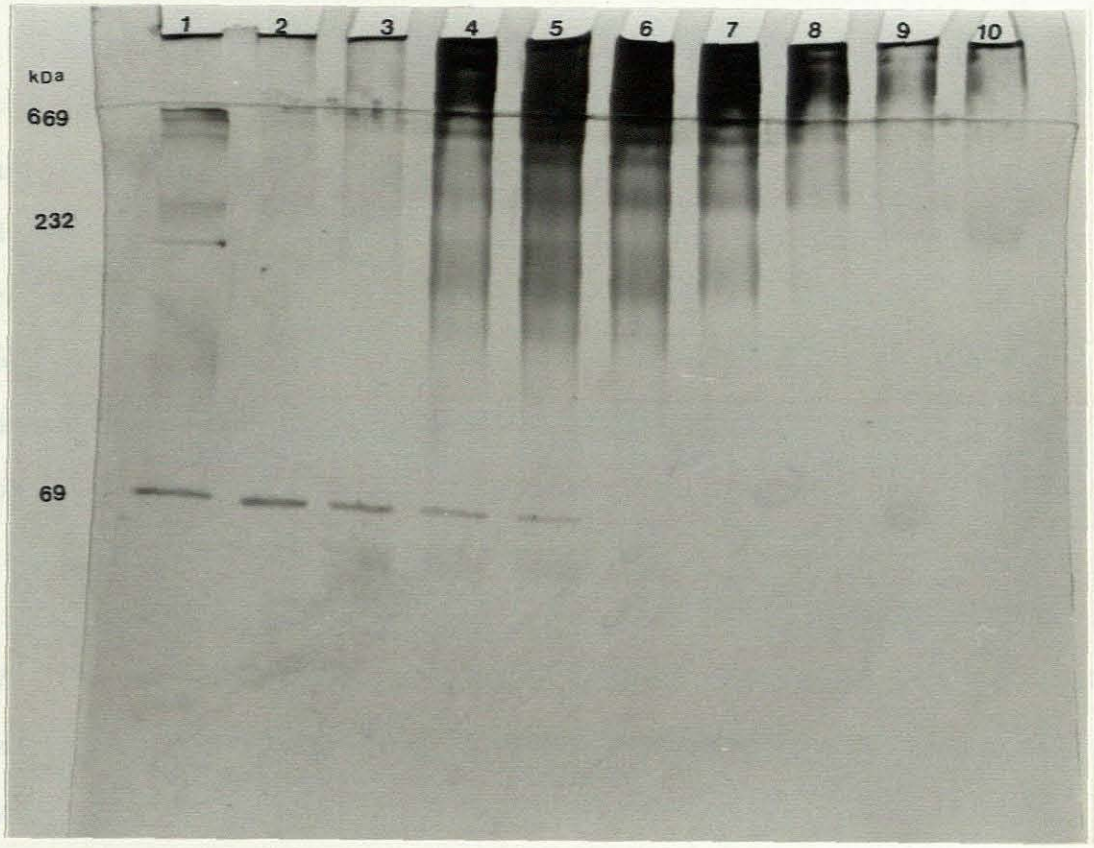



Plate 1c

Mucin rich fractions after the second equilibrium density gradient were pooled and subjected to a third fractionation. Fractions of the third equilibrium density gradient ultracentrifugation were electrophoresed on a 4-20% polyacrylamide gradient gel and stained for protein with the Biorad silver stain kit.

Lane 1: High molecular weight marker.

Lanes 2-10: Fractions 1-9.

## 4.2. Gel Filtration

### 4.2.1. *Sepharose 2B gel chromatography*

Gastric mucins were purified and isolated from solubilised mucus by two fractionations in a caesium chloride density gradient. To determine the ratio of mucin polymer to degraded subunits in mucins of all groups, freeze-dried samples were reconstituted in 0.2M NaCl:0.02% (w/v) sodium azide and subjected to Sepharose 2B gel filtration chromatography. Eluted fractions were assayed for mucin by the PAS reaction (Mantle and Allen, 1978) and for protein by the method of Lowry et al, (1951). The terms excluded peak or $V_o$, included peak or $V_i$ or total/near total volume peak or $V_t$ are used throughout the text to distinguish between polymeric mucins, degraded (subunits) with slightly smaller mucins and glycopeptides respectively.

The elution pattern representative for each of the four preparations (HD, PM, HGU and HCA groups), obtained by gel filtration on a Sepharose 2B column are shown in Fig. 13 (a-d).

Human transplant donor mucin (HD) eluted from the Sepharose 2B column (Fig. 13 a) as a small PAS positive excluded peak, (5% of the total carbohydrate material recovered), and a large heterogeneous included peak (85% of total mucin recovered), extending as a shoulder into the total volume (10% of total mucin recovered) of the column. Whilst a trace

of protein positive material was seen in the included volume of the column the bulk of the protein occurred as a sharp peak in the near total volume, coincident with the PAS positive shoulder. For the 6 HD samples analysed by Sepharose 2B gel filtration chromatography a mean value of 93.83% (s.e.m 1.91) was obtained for material that eluted in the included volume of the column (Tables 2 and 4).

Only 10% of PAS positive material of PM mucin eluted as an excluded peak in the void volume of a Sepharose 2B column (Fig. 13 b), the rest again eluting as a broad and polydisperse included peak (83% of total mucin recovered) that extended into the total volume (7% of total mucin recovered) of the column. Protein appeared in the void volume and spread across the included volume giving a heterogeneous multi-peaked appearance and a sharp prominent peak (55% of total protein recovered) in the total volume coinciding again with the PAS positive shoulder of mucin. Altogether 7 PM samples with a mean of 89.4% (s.e.m. 2.75) for low molecular weight material, were analysed by Sepharose 2B gel filtration chromatography (see Tables 2 and 5).

More mucin (22% of total mucin recovered) from the HGU group eluted as PAS positive material in the excluded volume of the Sepharose 2B column (Fig. 13 c). The included peak (70% of total mucin recovered) was again broad and polydisperse with the characteristic shoulder fractionating in the total volume of the column (8% of total mucin recovered). A trace

of protein in the form of a small sharp peak (13% of total protein recovered) was seen in the excluded volume and two more peaks in the near total and total volumes of the column eluted under the shoulder of mucin. Nine HGU samples were analysed by Sepharose 2B gel chromatography. Tables 2 and 6 show that HGU mucin contained ~84.8% (s.e.m. 3.6) low molecular weight material.

Fig. 13 d shows the Sepharose 2B profile for HCA mucin. A sharp PAS positive excluded peak (19% of total mucin) was followed by elution of the bulk (72% of total mucin recovered) of the material into the included volume of the column. The included peak displayed heterogeneity with a shoulder that extended into the total volume being more prominent than that of the other groups. The small sharp peak in the total volume accounted for 9% of the total mucin recovered. In the excluded volume of the column, protein accounted for 18% of the total mucin recovered while 27% eluted in the total volume of the column. This study included 14 HCA samples analysed by Sepharose 2B gel filtration chromatography (see Tables 2 and 7). It was found that low molecular weight mucins purified from HCA samples ranged from 66% to 94% with a mean of 83,92% (s.e.m.1.93%).

The amounts of degraded material in the HD (93.83% n=6) and HCA (84% n=14) groups differed significantly: $P<0.001$. No significant differences were seen between HD and PM, HD and HGU, PM and HCA or PM and HGU. These results suggested that

mucins from the PM, HCA and HGU groups were degraded to a similar extent.

4.2.2. *Sepharose 4B gel chromatography*

The similar extent of degradation for mucin of the PM, HGU and HCA groups on Sepharose 2B gel filtration made it difficult to establish the extent of this degradation in a single group as compared with the other groups. Material from each group (including the HD group) was therefore applied to a Sepharose 4B column which has a smaller pore size, making it possible to detect definite differences in the extent of degradation between the groups. Representative Sepharose 4B gel filtration profiles for mucin samples from each group are shown in Fig. 14 (a-d). HD mucin (Fig. 14 a) eluted as a sharp PAS positive peak of material (63% of total) in the excluded volume of the Sepharose 4B column, extending as a shoulder into the included volume and trailing into the total volume of the column. Protein also eluted as a sharp though smaller peak in the excluded volume of the column with material eluting as a smaller but wider peak in the total volume of the column.

Although PM mucin (Fig. 14 b) also displayed a sharp peak of mucin (55% of total material recovered) in the excluded volume, the peak in the included volume was more pronounced, eluting as a wide band of material trailing off into the total volume of a Sepharose 4B column. Again

protein eluted as a sharp excluded peak and a smaller and slightly wider peak in the total volume.

The human gastric ulcer Sepharose 4B gel filtration profile (Fig. 14 c) was quite similar to the HD in that the excluded material (56% Of total mucin recovered) eluted as a sharp peak while the included material eluted as a broad band which trailed off into the total volume of the column. Protein material was seen as a small peak in the excluded volume, as a "blip" in the included volume and as two identical small but wide peaks in the total volume of the column.

Mucin from the cancer patients displayed extensive heterogeneity (Fig. 14 d), eluting as a sharp PAS positive peak in the excluded volume (48% of total) followed by a series of sharp peaks of different sizes in the included volume and total volume of the Sepharose 4B column. There was hardly any protein material in the excluded volume but a wide Lowry positive peak was clearly evident in the included volume of the Sepharose 4B column.

These results suggested that the extent of degradation of mucins was greater in the HCA group than in any other group.

### 4.2.3. *Sepharose 4B gel filtration (of mucin recovered from the included volume of a Sepharose 2B gel filtration column)*

The Sepharose 4B gel filtration results shown above were of mucins obtained immediately after purification by density gradient centrifugation in a caesium chloride gradient. Here we describe the Sepharose 4B gel filtration analysis of mucins that had previously eluted in the included volume of a Sepharose 2B column.

PAS positive material eluting in the excluded, included and near total volumes of a Sepharose 2B gel filtration column were separately pooled. The 3 separate pools ($V_o$, $V_i$ and $V_t$) were dialysed repeatedly against distilled water, freeze-dried and stored at -20°C until required. The freeze-dried included volume material from a Sepharose 2B column of mucins from all groups was reconstituted in 0.2M NaCl:0.02% sodium azide (w/v) and prepared for gel chromatography on a Sepharose 4B column (Fig. 15 a-d).

Figure 15a shows that the HD mucin samples eluted mainly in the excluded volume (54%of total mucin recovered) of the Sepharose 4B column with the included material eluting as a shoulder into the near total volume of the column. No other PAS positive peaks were seen.

PM mucin, (Fig. 15 b) eluted as a definite peak in the excluded volume (43% of total mucin recovered) extending as a definite peak into the included volume of the column; this peak trailed off into the near total volume.

HGU mucin eluted as well defined peaks in both the excluded (41% of total mucin recovered) and included volumes of the Sepharose 4B column (Fig. 15 c). A small PAS positive peak was seen in the total volume of the column.

Material from HCA mucin (Fig 15d) eluted as a sharp peak in the excluded volume (37% of total mucin recovered). A large peak in the included volume extended into the total volume where no peaks were seen.

Again, these results gave an even more clear indication that degradation of mucins was highest in the HCA group followed by HGU and PM and HD.

4.2.4.SUMMARY

In this study, non-degradative methods developed in studies of pig mucus were used to purify mucins from human gastric mucus. Only surface mucus gel was studied and care was taken to exclude luminal contents and degraded mucus by draining the resected mucosa once the stomach had been opened along the greater curvature. Numerous studies on the size of pig gastric mucins have shown that low molecular weight (degraded) mucins have lost much of their viscous and gel forming properties. These degraded mucins are associated with a collapse of the mucus gel structure with subsequent impaired function (Snary et al, 1970). The relative amounts of polymeric and degraded mucins can be estimated by gel filtration chromatography (Younan et al, 1982). This technique uses gel forming, hydrophilic porous beads such as agarose to separate solutes on the basis of their molecular size. Openings and channels within the beads retard the smaller molecules while large molecules that cannot enter the beads flow along the spaces between them, thus eluting first. Sepharose 4B gel chromatography allows slightly degraded mucins to elute in the excluded volume of the column as the size of the beads is smaller than that of Sepharose 2B, which allows only molecules larger than $M_w 2x10^6$ to elute in the $V_o$ of the columnm.

Mucins from all groups (HD, PM, HGU and HCA), were found to be largely degraded (between 94% and 84%) mucins when

analysed by Sepharose 2B gel filtration (See Table 2). A small amount of PAS positive material, <10% of total carbohydrate in the HGU and HCA groups and ~18% in the HD and PM group, eluted in the total volume of the Sepharose 2B column. This component was often associated with protein positive material (Lowry) eluting as a peak in the same region (Fig. 13 a-d). It is a form previously described but not investigated further due to the small amounts obtained, nor was its presence accommodated in the concept of mucin structure (Younan et al, 1982). In the present study, material that eluted in the $V_t$ of a Sepharose 2B column was isolated and further analysed by gel electrophoresis (see Chapter 4.3).

A previous investigation into mucus from diseased and "normal" stomachs showed that "normal" mucus had significantly higher amounts of undegraded mucin, the source of the "normal" mucus being antrum resected at the time of a pancreatoduodenectomy (Younan et al, 1982); other investigators have reported the presence of large amounts (>50%) of polymeric mucins in human small intestinal mucus obtained from 12 hour post mortem samples (Mantle and Stewart, 1989).

Results for the control groups in our study were therefore unexpected, and since all mucins were purified in the presence of proteolytic inhibitors and not exposed to reducing agents before gel filtration chromatography, *in*

*vitro* degradation was highly unlikely. Because *in vitro* proteolysis was inhibited the large amount of degraded mucins in the HD (scraped within 40 minutes of death) and PM (scraped within 5 to 10 hours) groups implied the presence of a weaker gel on the surface of these mucosae due perhaps to a defect in biosynthesis or increased degradation *in vivo*. This degradation could be due to the accumulation of lysosomal proteolytic enzymes that are released during cell shedding. A greater amount of luminal mucus glycoprotein due to an increased shedding of mucus, has been observed during the development of erosions when experimental animals or humans are placed under stress (Glass and Slomiany, 1977). HD and PM subjects have been noted to die in traumatic circumstances eg. motor vehicle accidents, fatal stabbing or head injuries (See Table 4 and 5). Head injuries have been associated with the onset of gastric ulcer disease (Professor J. C. de Villiers Head of Neurosurgery UCT Cape Town, personal communication). In the PM group bacterial decomposition after death could play a role in the degradation of mucins. We assumed the absence of any gastric pathology for the control groups as tissue samples were not taken for histology because of operating theatre and mortuary regulations.

The use of denaturing solvents (e.g. GuHCl) to extract mucins has been reported to inhibit degradative enzymes and its use, together with proteinase inhibitors is strongly recommended for the extraction and purification of mucins (Carlstedt and

Sheehan 1984). However such solvents have been shown to perturb the native conformation of mucins; for example 4MGuHCl can cause aggregation of mucin molecules of $M_w 2x10^6$ to give soluble components of $M_w 8x10^6$ (Allen 1981). Therefore the use of denaturing solvents although avoided in this study is being considered in a separate study in future.

Since our Sepharose 2B gel filtration results showed hardly any difference between the groups samples were subjected to Sepharose 4B gel filtration to investigate any possible differences in size of degraded mucins between control and diseased states. Analysis of starting material on Sepharose 4B gel chromatography showed that while mucins were degraded in the order HCA> HGU> PM> HD all groups showed considerable $V_o$ peaks even though substantial $V_i$ peaks were still present (Fig. 14 a-d). Tables 2 and 3 show the distribution of PAS positive material on Sepharose 2B and Sepharose 4B columns respectively. Sepharose 4B gel filtration showed human donor mucins to be less degraded than mucins in the other groups; on the other hand Sepharose 2B gel filtration demonstrated that HD mucins had the least amount of polymeric mucin. The included peak ($V_i$) of HCA mucin on Sepharose 4B showed numerous peaks, an indication of the heterogeneity of that population of mucins, a characteristic not seen to this degree for mucins in the other groups.

When mucin eluting in the included volume of a Sepharose 2B column (degraded material) was chromatographed on a Sepharose

4B column, mucin from PM, HGU and HCA eluted mainly in the included volume while mucin obtained from HD specimens eluted mostly in the excluded volume of the column (Fig. 15 a-d). The glycoprotein recovered from the included volume of a Sepharose 2B column will primarily be of subunit size *i.e.*$5x10^5$ (Sellers et al 1988). However the broadness of the included peak on Sepharose 2B for all groups indicated the presence of a heterogenous population of mucins with the presence of material smaller than subunit size (glycopeptides) eluting in this peak. The greater elution of HD Sepharose 2B included mucins in the excluded volume of a Sepharose 4B column indicated that the extent of degradation of HD mucin was less than that for the other groups.

Our gel filtration results demonstrated that while mucins from all groups were substantially degraded, mucus from the diseased stomachs contained a greater population of low molecular weight material (Fig. 14 a-d, Fig. 15 a-d). Detailed studies on pig and human gastric mucus have shown structural similarity (Pearson et al 1980). Since the gel forming properties of pig gastric mucus are lost when substantial amounts of low molecular weight mucins are present, it follows that the presence of larger amounts of degraded mucins in our diseased samples would indicate a weak gel on the gastric mucosal surface. Such a gel would be less resistant and more vulnerable to the injurious effects of endogenous and exogenous aggressive factors acting on the mucosal surface.

**Table 2**

Distribution of PAS positive material after Seph 2B gel filtration (mean ± s.e.m.)

| Group | n | Polymeric mucin $V_o$ | Degraded mucin $V_i$ |
|---|---|---|---|
| HD | 6 | 6 | 94 (± 1.91) |
| PM | 7 | 11 | 89 (± 2.75) |
| HGU | 9 | 15 | 85 (± 3.60) |
| HCA | 14 | 16 | 84 (± 1.83) |

Glycoprotein was estimated by the PAS method of Mantle and Allen (1978).

**Table 3**

Distribution of PAS positive material after Seph 4B gel filtration

| Group | n | Pool of polymeric and slightly degraded mucin | Degraded mucin |
|---|---|---|---|
| HD | 1 | 63 | 37 |
| PM | 2 | 54 | 46 |
| HGU | 4 | 55 | 47 |
| HCA | 4 | 45 | 55 |

Glycoprotein was estimated by the PAS method of Mantle and Allen (1978).

**Table 4**

**Human transplant donors: History**

| Desig | Age | Sex | % LMW glycoprotein in gel | Cause of death |
|---|---|---|---|---|
| HD I | 30 | M | 95 | Cerebral vascular aneurysm |
| HD II | 54 | M | 85 | Subdural haemorrhage (CVA) |
| HD III | 22 | M | 99 | Motor vehicle accident |
| HD IV | 37 | M | 95 | Intracerebral bleeding |
| HD XI | 13 | M | 94 | Motor vehicle accident head injury |
| HD XII | 28 | M | 95 | |

**Table 5**

**Post-mortem: History**

| Desig | Age | Sex | % LMW glycoprotein in gel | Cause of death |
|---|---|---|---|---|
| PM I | 25 | F | 85 | Motor vehicle accident |
| PM II | 48 | M | 85 | Suicide |
| PM III | 30 | M | 90 | Stabbing |
| PM V | | M | 90 | Chest stab |
| PM VI | | F | 79 | Motor vehicle accident |
| PM XI | | | 97 | |
| PM XXII | | | 100 | |

**Table 6**

**Human gastric ulcer: History**

| Desig | Age | Sex | % LMW glycoprotein in gel | Histology |
|---|---|---|---|---|
| HGU I | 65 | F | 62 | Gastric ulcer, surrounding gastric mucosa normal |
| HGU IV | 53 | F | 78 | Diffuse superficial gastritis I.M.I. focal antral |
| HGU V | 69 | F | 78 | Superficial gastritis + GU |
| HGU VIII | 62 | M | 90 | I.M.II, I.M.III premalignant |
| HGU IX | 59 | F | 91 | Gastric ulcer; surrounding gastric mucosa normal |
| HGU XV | 46 | M | 90 | Gastric ulcer focal I M II |
| HGU XL | 59 | F | 84 | Metaplastic changes with a collagen infiltrate |
| XLI | 54 | M | 95 | Superficial gastritis |
| XLIII | 60 | M | 96 | Chronic active gastritis |

I.M. I - Intestinal Metaplasia Type 1
I.M. II - Intestinal Metaplasia Type 2

Table 7

*Human gastric cancer:* History

| Desig | Age | Sex | % LMW glycoprotein in gel | Histology |
|---|---|---|---|---|
| HCA I | 78 | F | 90 | Intestinal type (glandular) g.c. focal I.M.II |
| HCA III | 23 | F | 94 | Diffuse adenocarcinoma (poorly differentiated) |
| HCA IV | 79 | M | 89 | Diffuse poorly differentiated adenocarcinoma I.M.II patchy in areas |
| HCA VI | 70 | M | 66 | Early diffuse adenocarcinoma no mucosal involvement |
| HCA VIII | 51 | M | 81 | Differentiated intestinal type I.M.II |
| HCA IX | 59 | M | 82 | Intestinal type I.M.III |
| HCA XI | 68 | M | 81 | Intestinal type superficial gastritis no metaplasia |
| HCA XXXVIII | 55 | M | 92 | Adenocarcinoma intestinal and poorly differentiated |
| HCA XXXIV | ? | M | 87 | Intestinal type gastric adenocarcinoma |
| HCA XL | 68 | M | 83 | Multifocal diffuse adeno-carcinoma |
| HCA XLI | 52 | M77 | | Gastric carcinoma intestinal type |
| HCA XLII | 78 | F | 85 | Mucinous intestinal adeno-carcinoma |
| HCA XLIII | 47 | M | 79 | Intestinal adenocarcinoma |
| HCA XLVI | | | 89 | |

I.M. I - Intestinal Metaplasia Type I
I.M. II - Intestinal Metaplasia Type II

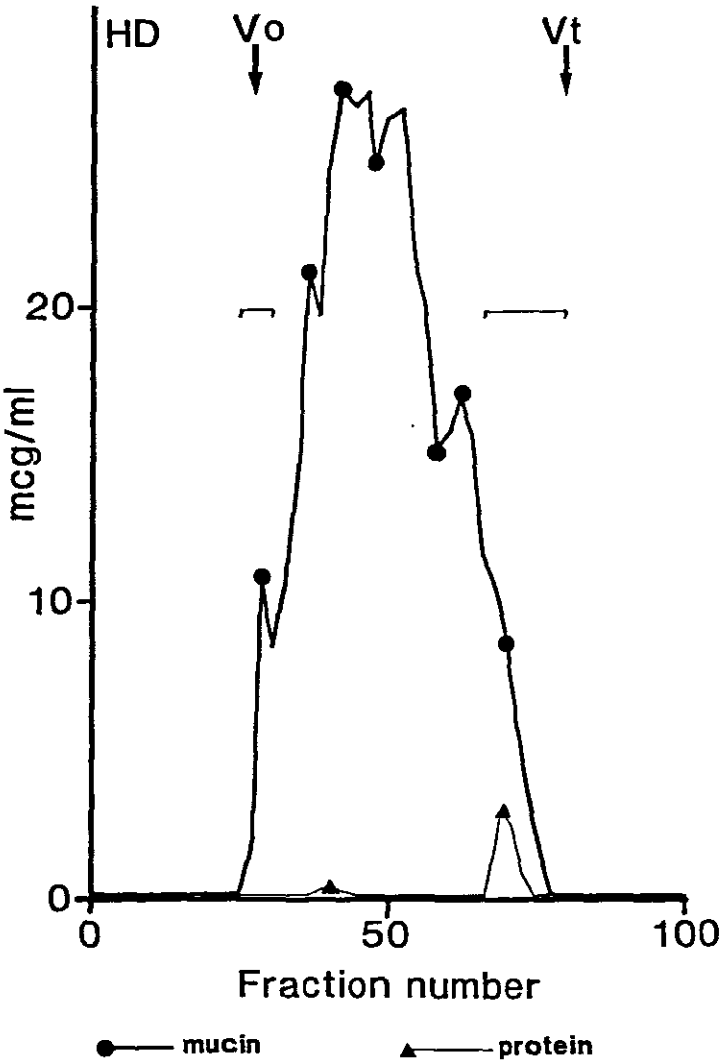



Figure 13a

Purified HD mucin (2mg/ml) was reconstituted in 0.2M NaCl:0.02% $NaN_3$ (w/v) and applied to a Sepharose 2B column (1,6x100 cm). Fractions were eluted with 0.2M NaCl:0.02% $NaN_3$. Fractions (~2.0ml) were assayed for mucin (PAS) and protein (Lowry). Fractions constituting the excluded ($V_o$) and near total ($V_t$) volumes were pooled as indicated by the bars. The fractions between the $V_o$ and $V_t$ constituted the included ($V_i$) volume.

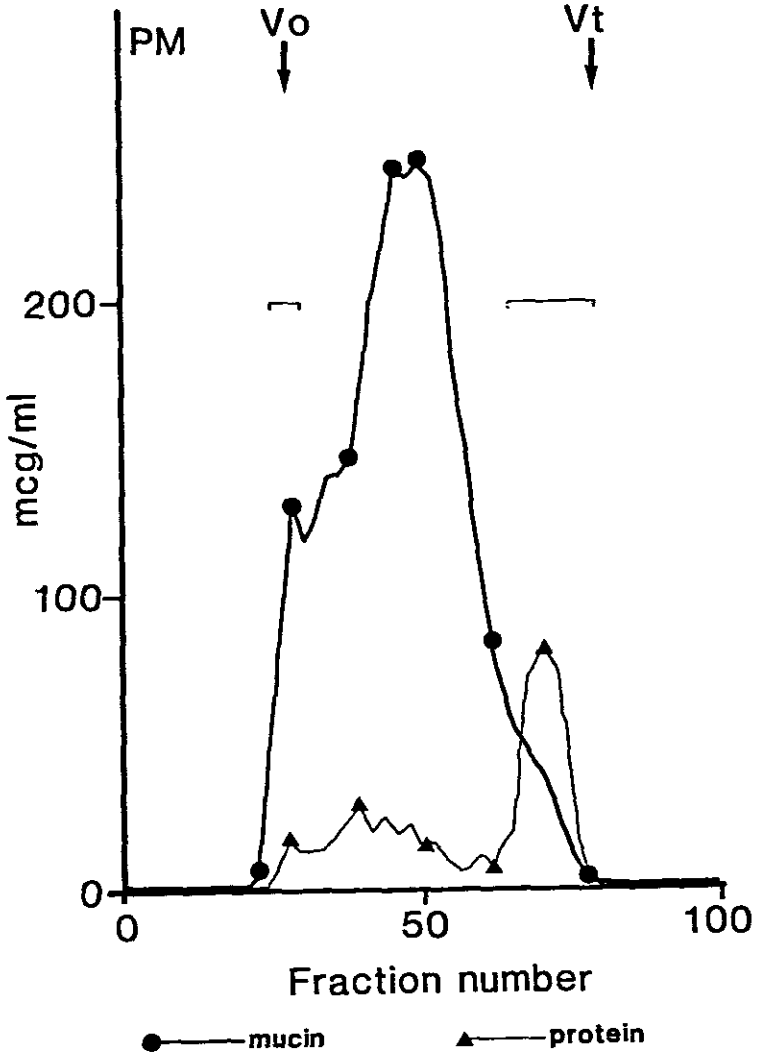



Figure 13b

Purified PM mucin (2mg/ml) was reconstituted in 0.2M NaCl:0.02% $NaN_3$ (w/v) and applied to a Sepharose 2B column (1,6x100 cm). Fractions were eluted with 0.2M NaCl:0.02% $NaN_3$. Fractions (~2.0ml) were assayed for mucin (PAS) and protein (Lowry). Fractions constituting the excluded ($V_o$) and near total ($V_t$) volumes were pooled as indicated by the bars. The fractions between the $V_o$ and $V_t$ constituted the included ($V_i$) volume.

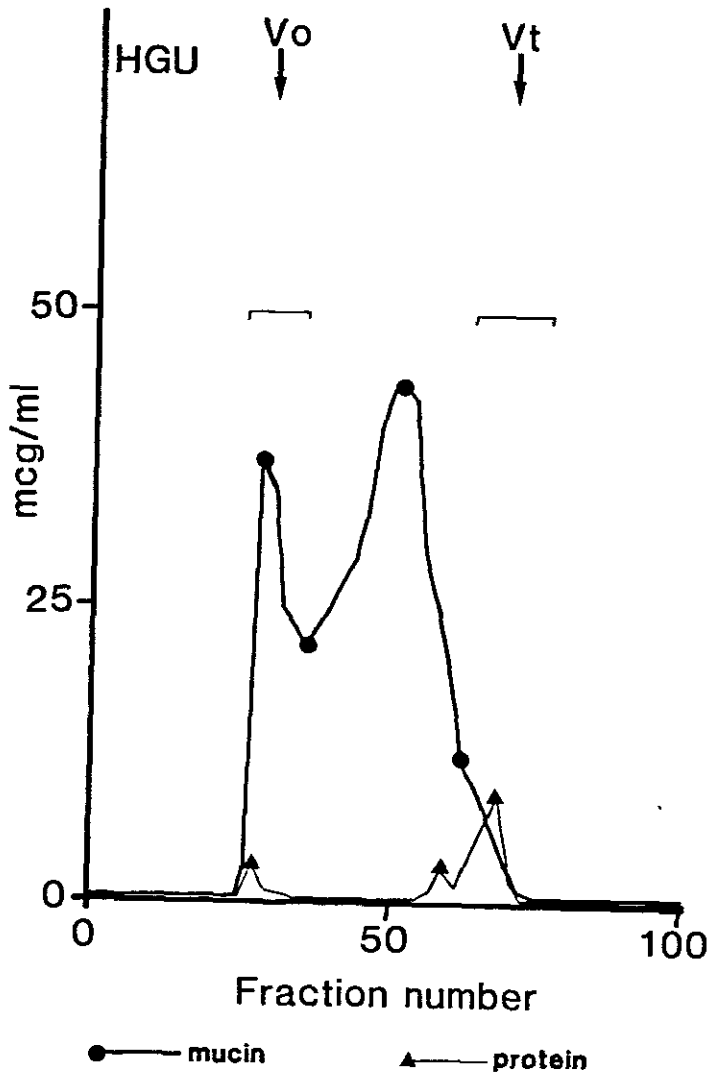



Figure 13c

Purified HGU mucin (2mg/ml) was reconstituted in 0.2M NaCl:0.02% $NaN_3$ (w/v) and applied to a Sepharose 2B column (1,6x100 cm). Fractions were eluted with 0.2M NaCl:0.02% $NaN_3$. Fractions (~2.0ml) were assayed for mucin (PAS) and protein (Lowry). Fractions constituting the excluded ($V_o$) and near total ($V_t$) volumes were pooled as indicated by the bars. The fractions between the $V_o$ and $V_t$ constituted the included ($V_i$) volume.

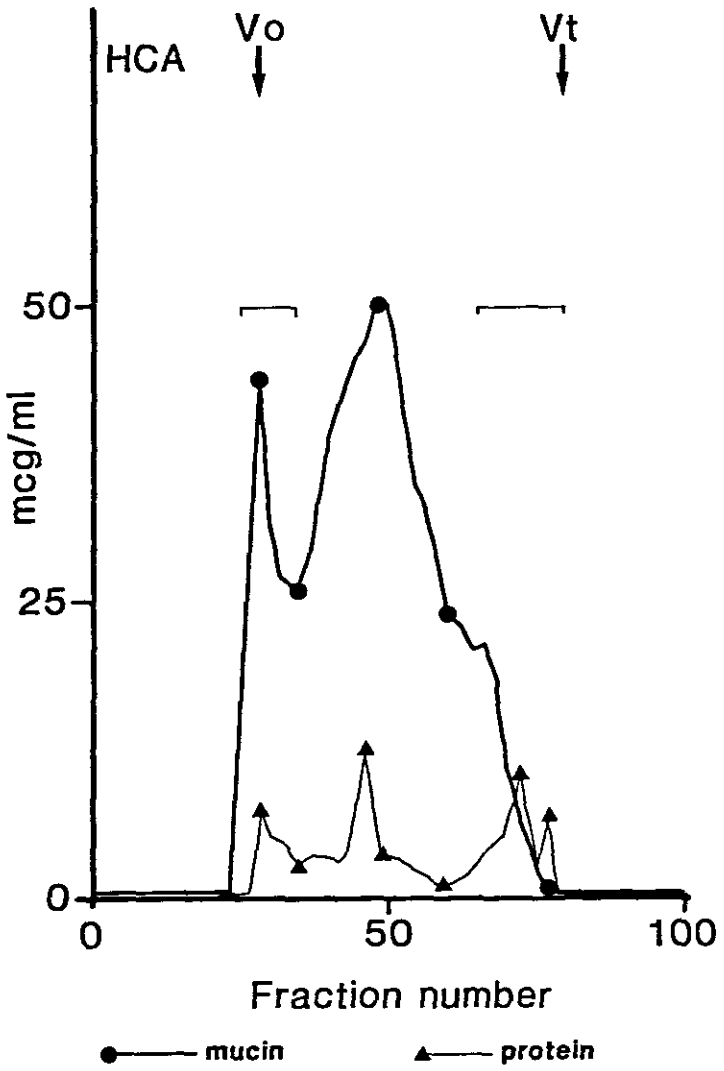



Figure 13d

Purified HCA mucin (2mg/ml) was reconstituted in 0.2M NaCl:0.02% $NaN_3$ (w/v) and applied to a Sepharose 2B column (1,6x100 cm). Fractions were eluted with 0.2M NaCl:0.02% $NaN_3$. Fractions (~2.0ml) were assayed for mucin (PAS) and protein (Lowry). Fractions constituting the excluded ($V_o$) and near total ($V_t$) volumes were pooled as indicated by the bars. The fractions between the $V_o$ and $V_t$ constituted the included ($V_i$) volume.

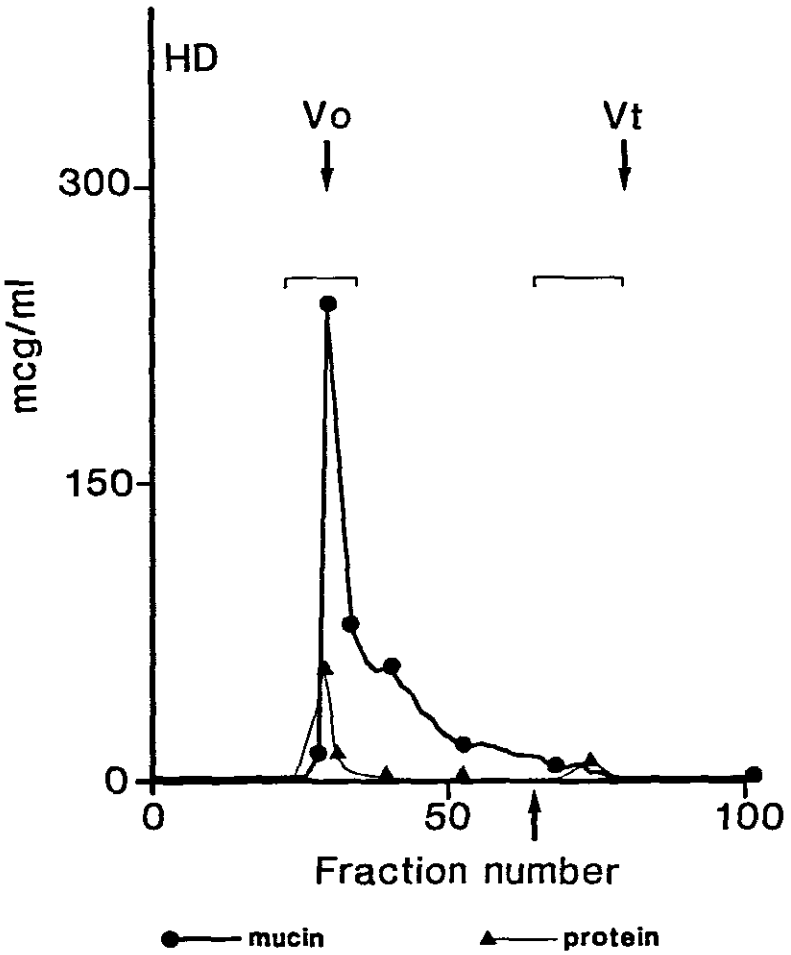



Figure 14a

Purified HD mucin (10mg/ml) was reconstituted in 0.2M NaCl:0.02% $NaN_3$ (w/v) and applied to a Sepharose 4B column (1,6x100 cm). Fractions were eluted with 0.2M NaCl:0.02% $NaN_3$. Fractions (~2.0ml) were assayed for mucin (PAS) and protein (Lowry).

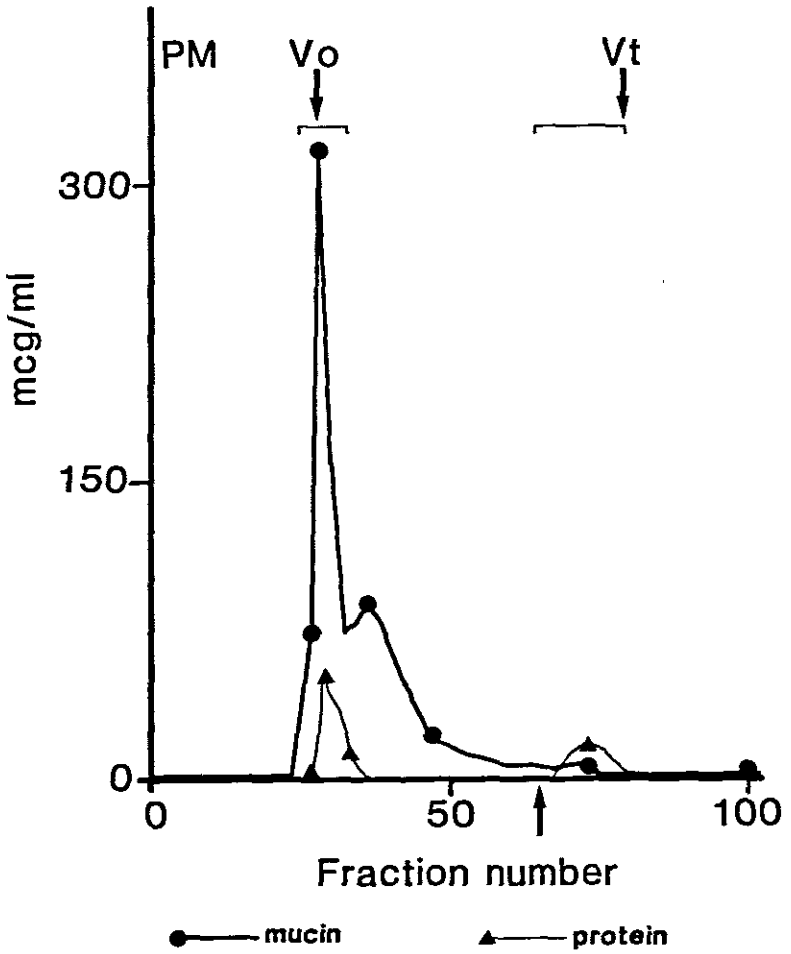



Figure 14b

Purified PM mucin (10mg/ml) was reconstituted in 0.2M NaCl:0.02% $NaN_3$ (w/v) and applied to a Sepharose 4B column (1,6x100 cm). Fractions were eluted with 0.2M NaCl:0.02% $NaN_3$. Fractions (~2.0ml) were assayed for mucin (PAS) and protein (Lowry).

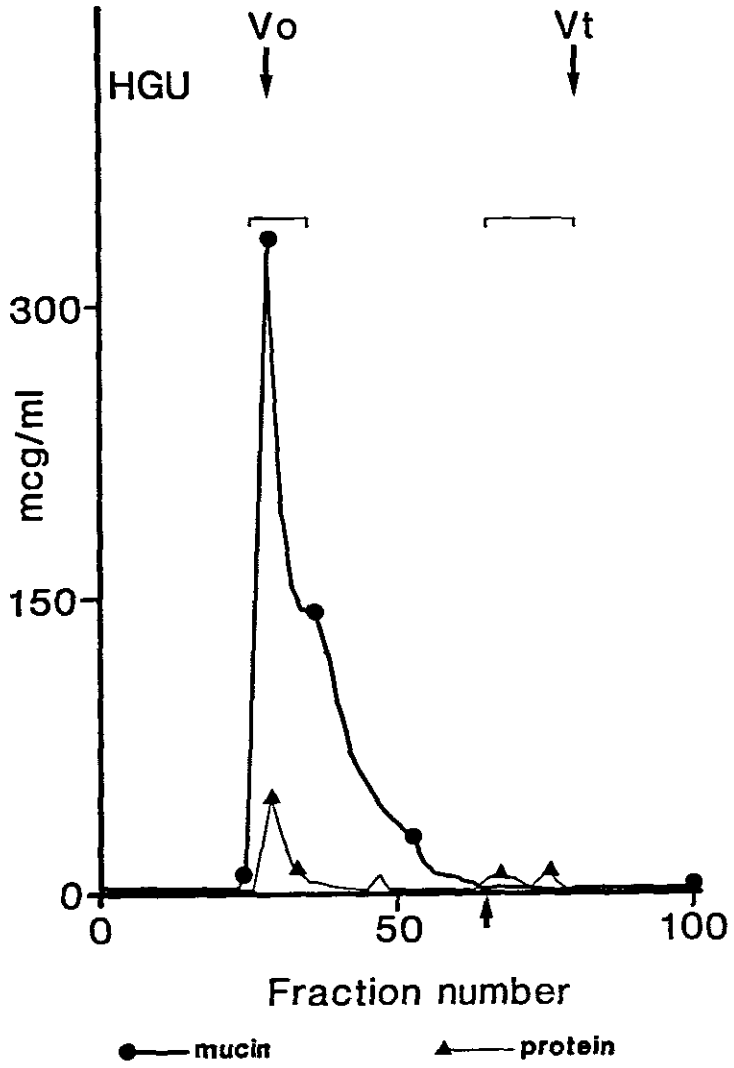



Figure 14c

Purified HGU mucin (10mg/ml) was reconstituted in 0.2M NaCl:0.02% $NaN_3$ (w/v) and applied to a Sepharose 4B column (1,6x100 cm). Fractions were eluted with 0.2M NaCl:0.02% $NaN_3$. Fractions (~2.0ml) were assayed for mucin (PAS) and protein (Lowry).

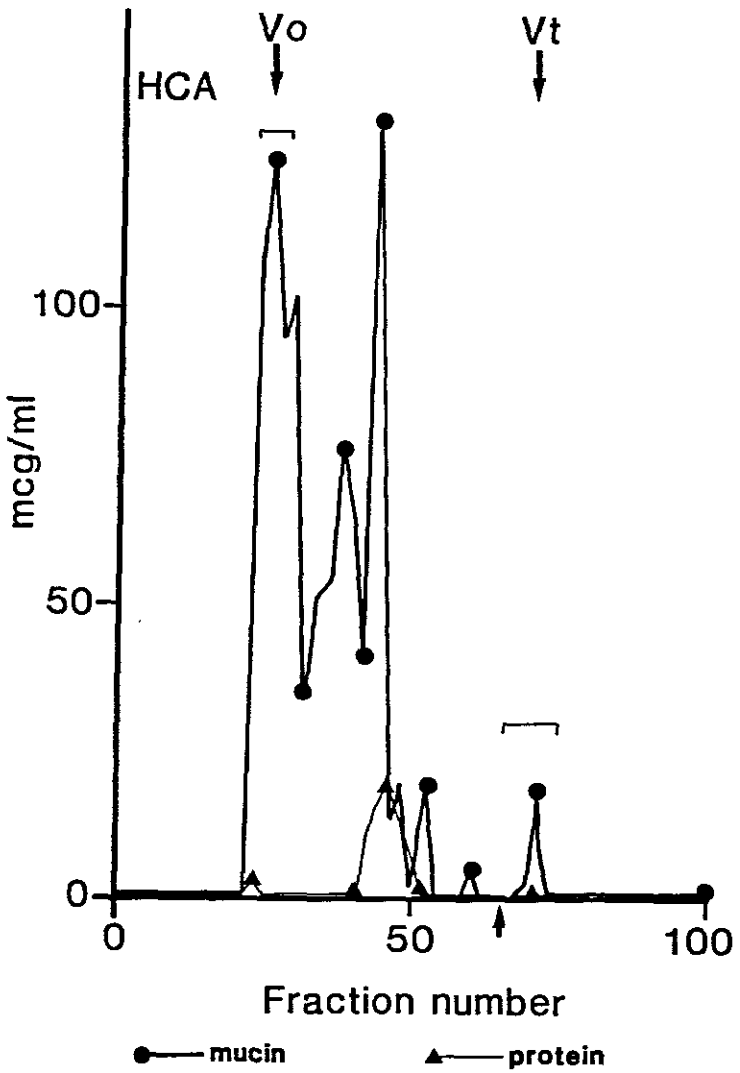



Figure 14d

Purified HCA mucin (10mg/ml) was reconstituted in 0.2M NaCl:0.02% $NaN_3$ (w/v) and applied to a Sepharose 4B column (1,6x100 cm). Fractions were eluted with 0.2M NaCl:0.02% $NaN_3$. Fractions (~2.0ml) were assayed for mucin (PAS) and protein (Lowry).

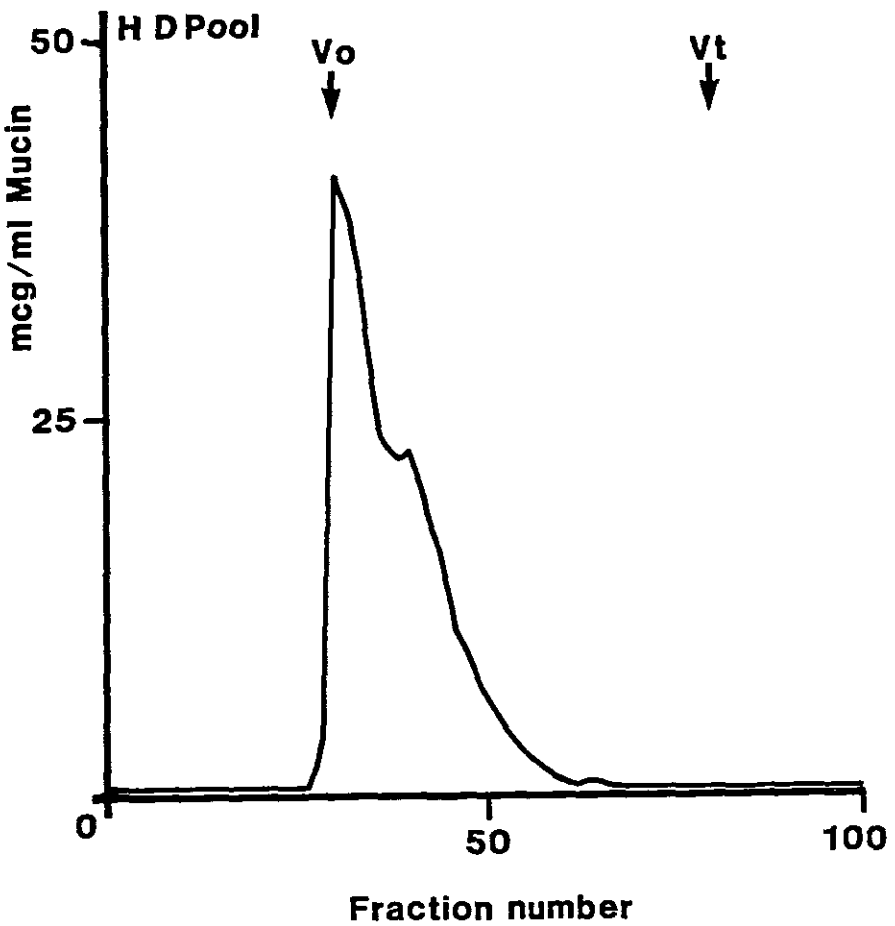



Figure 15a

Material harvested from the included ($V_i$) volume of a Sepharose 2B gel filtration column was dialysed and freeze-dried. The lyophilised material was reconstituted in 0.2M NaCl:0.02% sodium azide and rechromatographed on a Sepharose 4B gel filtration column.

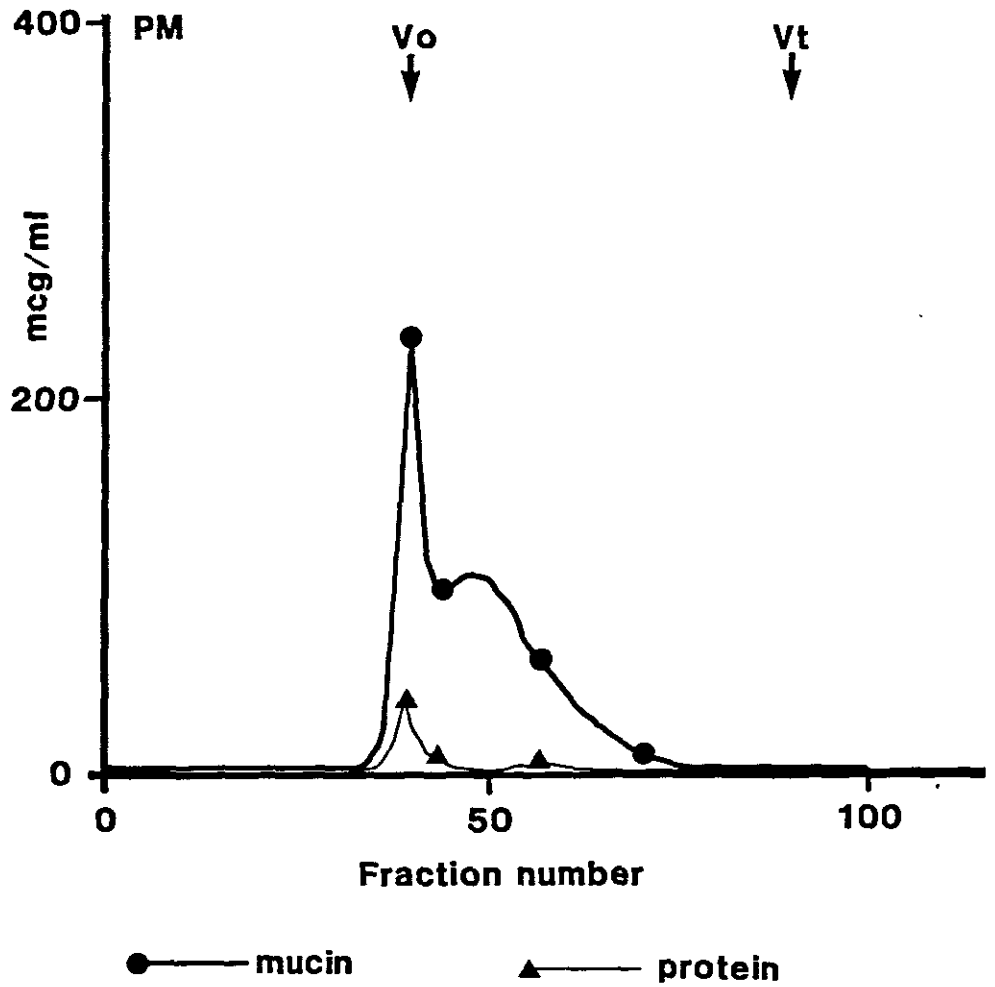



Figure 15b

Material harvested from the included ($V_i$) volume of a *Sepharose* 2B gel filtration column was dialysed and freeze-dried. The lyophilised material was reconstituted in 0.2M NaCl:0.02% sodium azide and rechromatographed on a Sepharose 4B gel filtration column.

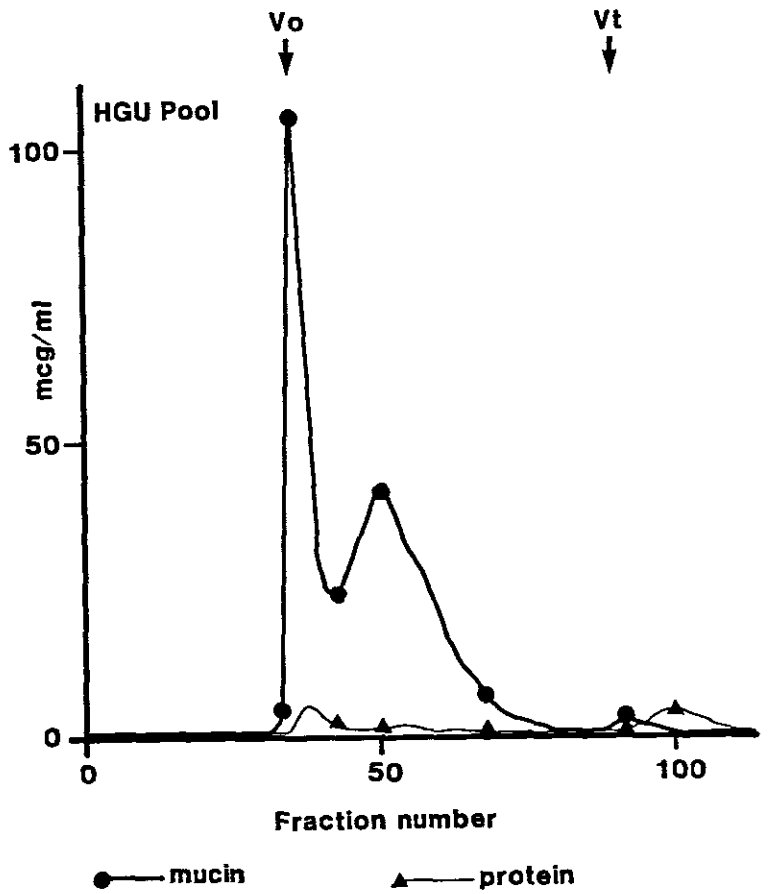



Figure 15c

Material harvested from the included ($V_1$) volume of a Sepharose 2B gel filtration column was dialysed and freeze-dried. The lyophilised material was reconstituted in 0.2M NaCl:0.02% sodium azide and rechromatographed on a Sepharose 4B gel filtration column.

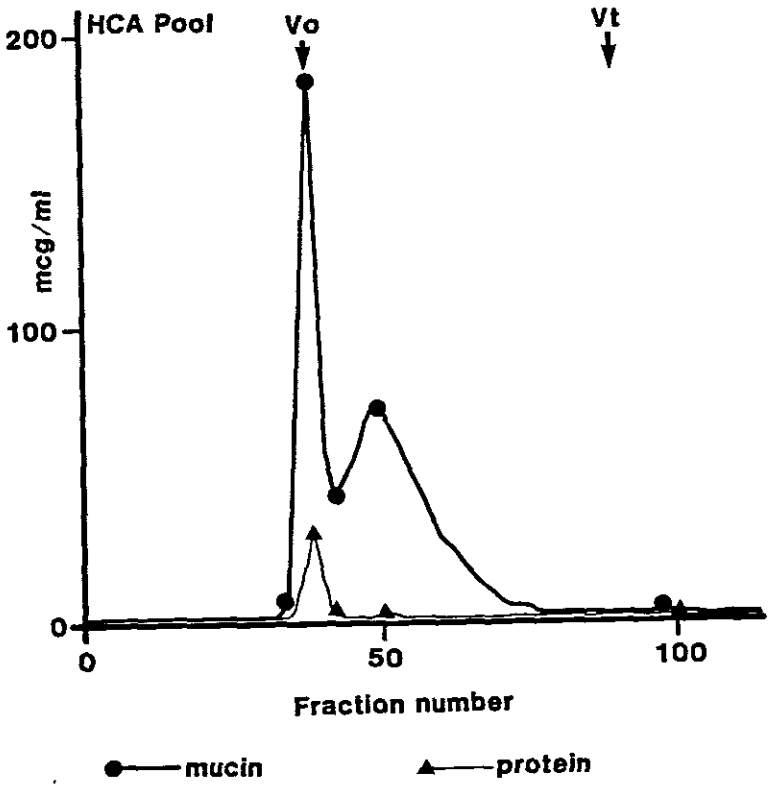



Figure 15d

Material harvested from the included ($V_i$) volume of a Sepharose 2B gel filtration column was dialysed and freeze-dried. The lyophilised material was reconstituted in 0.2M NaCl:0.02% sodium azide and rechromatographed on a Sepharose 4B gel filtration column.

## 4.3. Polyacrylamide Gel Electrophoresis

Polyacrylamide gel electrophoresis was performed on purified mucins isolated from mucus scrapings obtained from all groups in this study i.e. HD, PM, HGU and HCA. All samples were heated in 1% (w/v) sodium dodecyl sulphate (SDS) and electrophoresed before and after reduction in 0.2M 2-mercaptoethanol. Gels were stained for mucin by the PAS method of Dubray and Bezard, (1982), or for protein with $AgNO_3$ (Bio-Rad kit).

Purified human transplant donor mucin (HD), was electrophoresed on a 7,5% polyacrylamide gel and stained for protein (Plate 2, lane 2). A smear of intensely stained mucin material was seen at the top of the running gel, which faded at $M_w$~200kDa. Within this smear, bands were visible above and below $M_w$~200kDa. Very faint bands were seen at $M_w$~68kDa, 47kDa, 45kDa, 40kDa and 21.5kDa, which, in total most likely constituted <0.5% of the material applied to the gel. Mucin isolated from the mucus of a cancer patient (HCA) had a higher electrophoretic mobility, penetrating the running gel to a greater degree than the HD mucin (Plate 2, lane 3). Background staining for HCA mucin was even more intense than for HD mucin. Careful observation revealed the presence of bands of protein within the smear of $M_w$~200kDa. A well defined band at 68kDa was later confirmed to be albumin by Western blot analysis, with the use of anti-human albumin antibody (Plate 3, lane 2). The albumin had

associated with it, a large smear of heterogeneous material of the size range 50-65kDa, with an unusual negatively stained area within it (Plate 2, lane 3, arrow). Traces of bands were also visible between $M_w$~21.5kDa and 40kDa.

In order to determine whether the smear of material associated with the albumin was an artefact (due perhaps to caesium chloride being present in the HCA sample through incomplete dialysis after density gradient purification), HCA mucin was chromatographed on a Sephadex G-50 column and eluted with 0.2M NaCl:0.02% sodium azide (Fig. 16). The eluted fractions were assayed for mucin (PAS) and protein (Lowry). HCA mucin eluted as a well defined, PAS positive peak in the excluded volume of the column (Peak I) with a broad asymmetrical band of material, comparatively small in amount, in the included volume of the column (Peak II). Two protein positive (Lowry) peaks were seen; a short but wide peak in the excluded volume under the PAS positive peak and a heterogeneous wider band of material composed of three small peaks in the included volume of the column. Fractions comprising peak I ($V_o$)and peak II ($V_i$)were separately pooled, dialysed exhaustively against distilled water and freeze dried.

On SDS-PAGE, peak (I) mucin penetrated the running gel to a similar extent than before column chromatography on Sephadex G-50 (Plate 2, lane 4), with bands of material within the background (arrow), though not of a similar intensity. The

albumin-smear complex was again present with the negative stain within the smear even more pronounced (Plate 2, lane 4 arrow). On reduction in 0.2M 2-mercaptoethanol (Plate 2, lane 5), the electrophoretic mobility of the mucin increased and smears rather than well defined bands of material were visible within the background (arrows). Reduction in 0.2M 2-mercaptoethanol also released heterogeneous material at $M_w$~90kDa and a doublet was seen at $M_w$~65kDa. Lesser staining smears of varying intensity were seen below this band up to the end of the gel. Peak II material gave no bands on SDS-PAGE (Plate 2, lane 6).

Human cancer mucin which had been subjected to gel filtration on a Sephadex G-50 column was further characterised by SDS-PAGE on a 4-20% gradient gel stained for protein. Plate 2 (lane 8) shows that the mucin migrated about half the way into the gel with a very intense background, indicating that the heterogeneity of HCA mucin was greater than that shown on 7,5% SDS-PAGE. A well defined band was seen at $M_w$~68kDa, that being albumin. Immediately below this band a lighter smear associated with a negative staining region at $M_w$~65K was visible (Plate 2, lane 8 arrow). On reduction in 0.2M 2-mercaptoethanol the electrophoretic mobility and background of the mucin was greater and appeared more intense (Plate 2, lane 9). Well defined bands appeared at $M_w$~60kDa and $M_w$~70kDa and a range of lower molecular weight material could be seen for the reduced mucin up to the end of the gel (Plate 2, lane 9).

The human cancer mucin was applied to a 4-20% gradient gel under similar conditions as those above and stained for carbohydrate using the periodic acid Schiff base stain (PAS). A smear of glycoprotein positive material from the top of the running gel to about a third of the way down, was seen (Plate 2, lane 11). An intensely staining band at $M_w$~65kDa, coincident with the negative stain within the smear found on a gel stained for protein ($AgNO_3$) was seen. Reduction in 0.2M 2-mercaptoethanol (Plate 2, lane 12) increased the background and mobility of the mucin but did not change the amount or intensity of the very clear band at $M_w$~65kDa. These results suggested that the smear (including the negatively stained spot) seen at $M_w$~65kDa and associated with albumin on a gel stained for protein was strongly PAS positive on a gel stained for carbohydrate.

In order to establish whether this glycosylated factor, running very closely to albumin on SDS-PAGE was unique to the HCA group, mucin prepared from mucus scrapings of HD, PM HGU and HCA groups were subjected to 4-20% gradient polyacrylamide gradient gel electrophoresis. High molecular weight mucin was seen at the top of the stacking gel for all groups (Plate 4, lanes 1-9). A greater amount of intensely staining PAS positive material entered the running and stacking gel for PM (Plate 4, lane 1), HCA (Plate 4, lanes 3-6) and HGU mucin (Plate 4, lanes 7-9). Most of the intense PAS positive staining for the mucin of the HD group was seen at the top of the stacking gel and there was little migration

of PAS positive material into the stacking or running gel (Plate 3, lane 2). Mucin of the PM group displayed considerable electrophoretic mobility and intensity (Plate 4, lane 1) not unlike the mobility displayed by HCA mucin (Plate 4, lanes 3-6). The glycosylated factor was evident for HCA mucin (Plate 4, lanes 3-6) and HGU mucin (Plate 4, lanes 7-9), but not for mucin of the control (PM and HD) groups (Plate 4, lanes 1 and 2). Despite equivalent amounts of material applied to the wells, the intensity of the glycosylated factor varied between samples, with that in HCA mucin (Plate 4, lanes 3 and 5) being the faintest.

To determine whether this glycosylated component of $M_w$-65kDa seen for mucin of HCA and HGU groups was a fragment, originally part of high molecular weight intact mucin which diseased cells could not incorporate into a polymer during synthesis, control (HD), HCA and HGU mucin was electrophoresed on 4-20% SDS-PAGE before and after reduction in 0.2M 2-mercaptoethanol.

Plate 5 shows the comparison between HD, HGU and HCA mucins before and after reduction in 0.2M 2-mercaptoethanol (2-MeSH), on a gel stained for mucin with PAS. High molecular weight mucin was again seen at the top of the stacking gel for HD, HGU and HCA mucin (Plate 5, lanes 1, 2 and 3). Intensity and amounts of staining was highest for HD mucin (Plate 5, lane 1), whilst mobility and migration of the mucin into the gel (indicating heterogeneity of size of the mucin

and greater degradation) was evident for HCA mucin (Plate 5, lane 3). The glycosylated component of $M_w$~65kDa seen again for HGU and HCA (Plate 5, lanes 2 and 3), was absent in the HD sample (Plate 5, lane 1). Reduction of mucins of all groups in 0.2M 2-mercaptoethanol showed the release of large amounts of material of different size penetrating almost halfway into the gel for HD mucin (Plate 5, lane 4), with a similar though less mobile trend for HGU mucin (Plate 5, lane 5). Little release and migration of material occurred for the HCA mucin after reduction in 0.2M 2-mercaptoethanol, indicating that lesser amounts of intact mucin were originally present in this group (Plate 5, lane 6). HD mucin (Plate 5, lane 1) followed by HGU mucin (Plate 5, lane 2) were originally larger disulphide bonded aggregates. The $M_w$~65kDa glycosylated component for the HGU and HCA samples (Plate 5, lanes 2 and 3 respectively) did not change in appearance and amount, and was still absent for HD mucin (Plate 5, lane 4), after reduction in 0.2M 2-mercaptoethanol.

Plate 6 compares mucin purified from post mortem (PM) mucus with HCA and HGU mucin before (lanes 1, 2, and 3) and after (lanes 4, 5, and 6) reduction in 0.2M 2-mercaptoethanol, separated on a 4-20% gradient gel stained for mucin with PAS. The electrophoretic mobility, penetration and spread of HCA and (Plate 6, lane 2) and HGU mucin in the gel (Plate 6, lane 3) was greater than PM mucin (Plate 6, lane 1). At $M_w$~65kDa a PAS positive band can be seen in HCA and HGU mucin (Plate 6, lanes 2 and 3) but not in PM mucin (Plate 6, lane 1).

Upon reduction in 0.2M 2-mercaptoethanol post mortem mucin (Plate 6, lane 4) penetrated the gel much more displaying a high background. This suggested that like HD mucin, most of the original material was of a larger size than mucins from HGU and HCA mucus, which also released material, though it was of a lesser amount. The glycosylated component did not appear after 0.2M 2-mercaptoethanol treatment for the PM mucin (Plate 6, lane 4) but was again seen for HCA and HGU mucin (Plate 6, lanes 5 and 6).

To determine the size of the glycosylated factor more carefully, two separate aliquots of mucin from HD, HGU and HCA groups were loaded onto a 4-20% gradient gel and stained for protein and carbohydrate for side by side observation. In plate 7, lanes 1-4 were stained for protein ($AgNO_3$) and lanes 5-7 were stained for mucin (PAS). When samples were stained for protein, smears of intensely stained material were seen in the stacking gel for HD (lane 1), HCA (lane 2) and HGU mucin (lane 3). In the running gel HD mucin (lane 1) ran as an intense smear to about a third of the way, fading before the lightly stained and characteristic albumin band seen at $M_w$~68kDa. HGU mucin (lane 3) travelled through the gel in much the same way as HD mucin, but the intensity of the smear faded only slightly before the albumin band. A light staining smear was present below and close to the albumin band. The intensity of the smear for HCA mucin (lane 2) lasted almost all the way to a prominent albumin band and just below the latter, a definite smear could be seen

(Plate 7, lane 2 arrow). HCA mucin showed low molecular weight material, between 25kDa and 10kDa, when stained for protein with $AgNO_3$ (Plate 7, lane 2).

Aliquots of mucin samples of the same groups (HD, HCA and HGU) (plate 7, lanes 5-7), were stained for carbohydrate with PAS. Intensely stained material could be seen for all samples in the stacking gel and at the top of the running gel. HD mucin (Plate 7, lane 5) travelled further into the gel than HCA (Plate 7, lane 6) and HGU (Plate 7, lane 7) mucin. While smears of material for HCA and HGU were not as electrophoretically mobile as HD mucin, they did display well defined bands at $M_w$~65kDa (Plate 7, lanes 6 and 7). No bands were seen at this molecular weight for HD mucin (Plate 7, lane 5) when samples were stained for mucin. This gel confirmed that bands of $M_w$~68kDa seen on gels stained for protein and bands of $M_w$~65kDa seen on gels stained for mucin, exhibited a small but definite difference in molecular size between a protein and an associated (bound) carbohydrate fragment.

Mucin from all groups displayed upon elution on Sepharose 2B gel filtration a protein positive peak under a PAS positive shoulder near the total volume of the column (see gel filtration results). Whilst the carbohydrate and protein content of this peak varied between samples of all groups, it nevertheless was always evident after Sepharose 2B gel filtration (Fig. 13 a-d). Fractions under this peak were

pooled, dialysed exhaustively against distilled water and freeze-dried. Because of the minute quantities recovered for each individual sample, it became necessary to further pool samples within each group i.e. HD, PM, HGU and HCA in this way harvesting sufficient quantities of near total volume peak material.

SDS-PAGE analysis (4-20% gradient gel) of a pool of HCA near $V_t$ material followed by staining with $AgNO_3$ for protein confirmed the presence of the albumin band associated with the negative stain below it (Plate 8, lane 1 arrow). An aliquot of the same sample electrophoresed on the same gel gave an intensely staining PAS positive band (Plate 8, lane 2) coinciding with the negative staining smear on the protein stain at $M_w$~65kDa.

A comparison of pooled (more than 1 patient) near $V_t$ material of all groups on SDS-PAGE followed by staining for carbohydrate with PAS again gave a prominent band at $M_w$~65kDa for the pooled HGU (Plate 9, lane 3) and pooled HCA (Plate 9, lane 4) groups, and in this instance three narrow lightly stained bands for the pooled HD group (Plate 9, lane 1) and traces of two bands for the pooled PM group (Plate 9, lane 2) all in the region of $M_w$~65kDa. In lane 1 pooled HD mucin entered the running gel and migrated as an intense smear for about a third of the way into the gel. The mucin stained intensely in the stacking gel and at the top of the running gel, faded as it penetrated the gel and at $M_w$-65-69kDa three

lightly stained bands were seen Plate 9, lane 1). The electrophoretic mobility of the pooled PM mucin was similar to that of the pooled HD mucin but the background staining was not as intense. Two faintly stained bands can be seen at Mw~65kDa (Plate 9, lane 2). The pooled HGU mucin stained intensely in the stacking gel and in the running gel displaying considerable electrophoretic mobility. At Mw~65kDa a well defined and heavily stained band is seen (Plate 9, lane 3). Pooled HCA mucin showed a similar though less intense pattern of migration of mucin into the stacking and running gel with a strongly PAS positive band at $M_w$~65kDa (Plate 9, lane 4).

Finally, to exclude the possibility that the glycosylated component (obtained by fractionation on Sepharose 2B as described above), consistently present for HCA and HGU mucin, was albumin staining artefactually with PAS on these gels, pure albumin, at high loadings of 500ug (plate 10, lane 3) and 1000ug (plate 10, lane 4) were stained for carbohydrate. Only traces of a band were found at $M_w$~68kDa. In contrast, less than 100ug of the glycosylated factor, harvested on a Sepharose 2B gel filtration column and mixed with 500ug albumin (Plate 10, lane 2) gave an intense PAS positive stain on SDS-PAGE.

Mucins from PM, HGU and HCA mucus were chromatographed on a preparative Sepharose 4B column eluted with 0.2M NaCl:0.02% sodium azide. The eluted fractions in the excluded, included

and near total volumes were pooled as 3 separate peaks, (as shown in Fig. 14 a-d) dialysed against distilled water and freeze dried. The mucins isolated from the included volume of a Sepharose 4B column were reconstituted in sample application buffer containing SDS and subjected to SDS-PAGE. The gel was stained for carbohydrate (Plate 11) and the spectrum of degradation is shown by the increasing depth of migration through the stacking gel i.e. PM (lane 1) <HGU (lane 2) <HCA (lane 3). There is evidence of HCA mucin entering the top of the running gel when harvested from the included volume of a Sepharose 4B column (lane 3), indicating that the extent of degradation of mucin was highest in this group compared to the HGU group which in turn was more degraded than that of the PM group.

Gastric juice from PM, HGU and HCA patients was homogenised in phosphate buffer pH 6.5, containing proteolytic inhibitors and centrifuged at 10,000 rpm for 60 minutes; the supernatant was strained through glass wool, exhaustively dialysed against distilled water and subsequently freeze-dried.

Duplicate samples of crude gastric juice preparations were electrophoresed on a 4-20% gradient gel and stained for protein ($AgNO_3$) and carbohydrate (PAS) (Plate 12). PAS staining for PM gastric juice was positive at the top of the stacking gel (Plate 12, lane 1) whilst a spread of protein positive material upon $AgNO_3$ staining was seen in the stacking gel and the top of the running gel

(Plate 12, lane 2). No other bands were seen in either lanes 1 or 2 (Plate 12).

Gastric juice material from the HGU group (Plate 12, lane 3) gave a spread of material intensely stained with PAS. The sample penetrated the running gel about a quarter of the way but no other bands were seen. When the same sample was stained for protein ($AgNO_3$) (Plate 12, lane 4), protein positive material was seen at the top of the stacking gel. Careful observation showed that the sample had entered the running gel. No other bands were seen in lanes 3 or 4. Gastric juice from a HCA patient stained for carbohydrate with PAS was seen at the top of the stacking gel (Plate 12, lane 5), with some material entering the running gel and PAS positive bands seen at $M_w$~440kDa and at $M_w$~65-68kDa. Plate 12 (lane 6) showed the same HCA gastric juice staining very intensely for protein at the top of the gel $M_w$~440kDa and fading slightly at $M_w$~140kDa. At $M_w$~68kDa a strongly stained broad band (that being albumin) is followed by 3 narrow, well defined but lighter staining bands.

The greater electrophoretic mobility (and therefore smaller size) of mucins especially on PAS staining in the HGU and HCA groups compared to the PM group is in keeping with what has been found for mucins extracted from crude gel scrapings off the mucosal surface. The presence of the albumin associated glycosylated factor in the gastric juice of a cancer patient should be noted.

Gastric juice from a HCA patient was applied to a Sepharose 2B gel filtration column and eluted with 0.2M NaCl:0.02% sodium azide (Fig. 17). Material eluting as excluded, included and near total volume peaks were separately pooled, dialysed exhaustively against distilled water and freeze-dried. On SDS-PAGE analysis (Plate 13) stained for mucin, the excluded volume material (lane 2) was found on the top of the stacking gel, the included volume material stained more intensely at the top of the stacking gel and entered the running gel (lane 3) and the near total volume material (lane 4) entered the running gel and gave an array of bands. A lightly stained smear of PAS positive material with a narrow band immediately beneath, was seen at $M_w$~65-68kDa, indicating that this factor, present in gastric juice of a cancer patient only, can be harvested on a Sepharose 2B column from a near $V_t$ peak, like its counterpart in the gastric mucus gel.

4.3.1. SUMMARY

Gel electrophoresis is used successfully for most protein characterisation studies. Its application to mucus glycoproteins has not been as successful though, the reason being the lack of migration by the mucins through the gels and the smear characteristics of heavily glycosylated molecules compared to the distinct and well defined appearance of protein bands (Holden et al, 1971). An in-depth study by Holden et al, (1971), showed that gel eletrophoresis gave some indication of relative molecular weight, polydispersity and purity even when using small (<1mg) amounts of mucin samples. The carbohydrate-protein composition could be assessed with the use of specific staining techniques. We have thus employed SDS-PAGE to characterise mucins purified from mucus obtained from individuals with a) known gastric pathology (HGU) and (HCA) and b) "normal" gastric mucosa (HD and PM). The gels were stained for mucin using the PAS reaction (Dubray and Bezard, 1982) or for protein using silver nitrate (Bio-Rad kit).

Our gel filtration results on Sepharose 2B showed similar profiles for all groups in this study. However our SDS-PAGE results show HD mucins to be less degraded than those of the PM, HGU and HCA groups (Plate 4). This observation is perhaps in keeping with our Sepharose 4B results which showed that while HD mucin was degraded, the degradation was not as extensive as in the other groups. The excluded PAS positive

material of HD mucin on a Sepharose 4B column accounted for 63% of the total PAS positive material recovered, while that of the other groups (PM, HGU and HCA) accounted for less.

PM mucin was shown to be more electrophoretically mobile than HD mucin (see Plate 4) giving high background staining not dissimilar to HCA and HGU mucin. This raised questions about the validity of using PM mucin as a control considering that material is only available 5-10 hours after death due to stringent mortuary regulations which limit the availability of material, especially within 10 hours. During this time corpses are stored at room temperature and the possibility of endogenous degradation through bacterial contamination, autolysis and cell shedding cannot be ruled out. There are additional difficulties associated with using PM stomachs as a source of mucus for a control in any study; many of the available stomachs are those of subjects who have died traumatically (usually severe head injuries) in alcohol related incidents (see Table 5). Histological examination of the gastric mucosa of the cadavers is necessary to exclude early or advanced gastric disease, something we were unable to do since permisssion had not been granted for biopsies to be taken. Furthermore, the volume of scrapable mucus on the surface of the mucosa of PM stomachs is at least six fold greater than that found on HD gastric mucosae. It has been shown in pigs that an increased amount of mucus on the mucosal surface is correlated to an increased storage time of cadavers (Mall, personal communication). It is possible that

spontaneous contractility of the stomach after death would release stores of preformed intracellular mucus; this mixing of intracellular and surface mucus would further invalidate a comparison between PM mucus and that of other groups, making post mortem mucus specimens an even poorer control in any study.

Mucus from transplant donor subjects is usually fresher (within 1 hour) but, like PM stomachs, not frequently available (this laboratory obtained 10 samples within 2 years). Gel filtration studies have, unexpectedly, shown these mucins to be degraded; again this could be related to traumatic deaths (mostly head injuries) (see Table 4). An association has been reported between severe head injuries and gastric ulceration (Professor J.C. de Villiers, personal communication). Again careful investigation is needed to determine how normal these stomachs are. Pepsin levels after a head injury could be investigated since an increase in pepsin activity could result in increased degradation of the mucins on the surface of the gastric mucosa. Despite the similar Sepharose 2B gel filtration profiles of gastric mucins between the control and the diseased groups in this study, SDS-PAGE analysis have shown that both control groups, but mainly the HD group had more large sized mucins. In some cases these mucins hardly penetrated the stacking gels and it is conceivable that they were washed away during the fixing and staining process of these gels.

It is noted that a PAS positive band at $M_w$~65kDa constantly appeared in samples from the HGU and HCA groups. Trace amounts of this glycosylated factor was seen only when a pool of control samples (i.e. HD pool or PM pool) was electrophoresed and then stained for mucin (Plate 9). The factor was entirely absent in mucus and mucin of other PM and HD samples. It has been confirmed by Western blot analysis that this PAS positive material is associated with albumin. That the albumin resisted dissociation from the mucins in a 3.5M caesium chloride density gradient indicated that this association is strong; the fractionation of this carbohydrate complex with mucins suggested that it was heavily glycosylated. .The complex is however dissociated from mucins by heating at 100°C in SDS for 2 minutes. It is known that there is an increased leakage of serum albumin into the stomach during an actively bleeding ulcer. Albumin is present in excessive amounts in the stomachs of patients with gastric cancer (Creeth, 1978). Whether the conformation of this albumin is altered in diseased states in a way that enhances the strong association with a glycoprotein fragment as shown here, is not known. It is also not known whether the carbohydrate portion of the complex is a fragment of mucin secreted by a malignant cell or whether it is a product of endogenous proteolysis due to bacterial contamination in an environment of pH~5. Coldrey, (1987) reported that the gastric juice of patients with gastric cancer has a pH of about 5, making it likely that bacterial contamination occurs in patients with gastric cancer. However it is unlikely that

this glycosylated factor is linked to the mucins by disulphide bonds, since treatment with 0.2M 2-mercaptoethanol did not increase its release on SDS-PAGE.

The appearance of this complex in mucus secretions of all individuals with cancer of the stomach raised early hopes in this project, of it being a marker for malignancy. Its presence (later detected) in mucins from HGU patients reduced its potential as a marker for malignancy although, it should be emphasized that the mucosae of some of the patients with diagnosed ulcers, were declared premalignant upon subsequent histological examination of tissue taken at the time of surgery (see Table 6).

The absence of this characteristic stain for the albumin-glycoprotein complex at Mw~55-65kDa on SDS-PAGE for mucins from a cancer patient used to show purification after CsCl density gradient centrifugation, should be noted. This sample was from a cancer patient who was diagnosed as having a leishomyosarcoma, different to the epithelial type carcinoma diagnosed for all other patients in this study (Table 7).

It has been confirmed that the complex is not an artefact of albumin being stained with PAS. It has been shown that it is easily harvested in the near total volume of a Sepharose 2B gel filtration column even though the yield is minimal and its purity incomplete as shown by the presence of a mild

background associated with the factor on SDS-PAGE. Further purification of this factor with the employment of other methods is being considered, since fractionation by gel chromatography on Sepharose 2B does not offer complete purity. A small but distinct difference in size between the albumin and the carbohydrate portion of the complex has been shown by SDS-PAGE analysis. The presence of this factor in the gastric juice of a patient with gastric cancer, though a very preliminary finding is an encouraging one. More suitable controls will be available in studies of gastric juice in which the latter could be obtained easily through orogastric-intubation in normal and diseased states.

It has been reported that mucins in gastric juice are unsuitable for study since they are largely degraded as a result of peptic activity on the adherent mucus gel (Allen, 1981). In a separate ongoing study we have found that considerable amounts of polymeric mucin (undegraded) is present in the gastric juice of a patient whose ulcer had healed after the administration of sucralfate (Mall, unpublished data). It was confirmed that this polymeric mucin was not merely a non-covalent aggregate of degraded mucin subunits as it was not dissociated by heating in SDS at 100°C for 10 minutes. The ideal source of mucus for use as a control would undoubtedly be a live normal subject, but to obtain adherent mucus gel would be difficult. However, in the light of the present gastric juice results investigation of gastric juice from normal subjects is a feasible option.

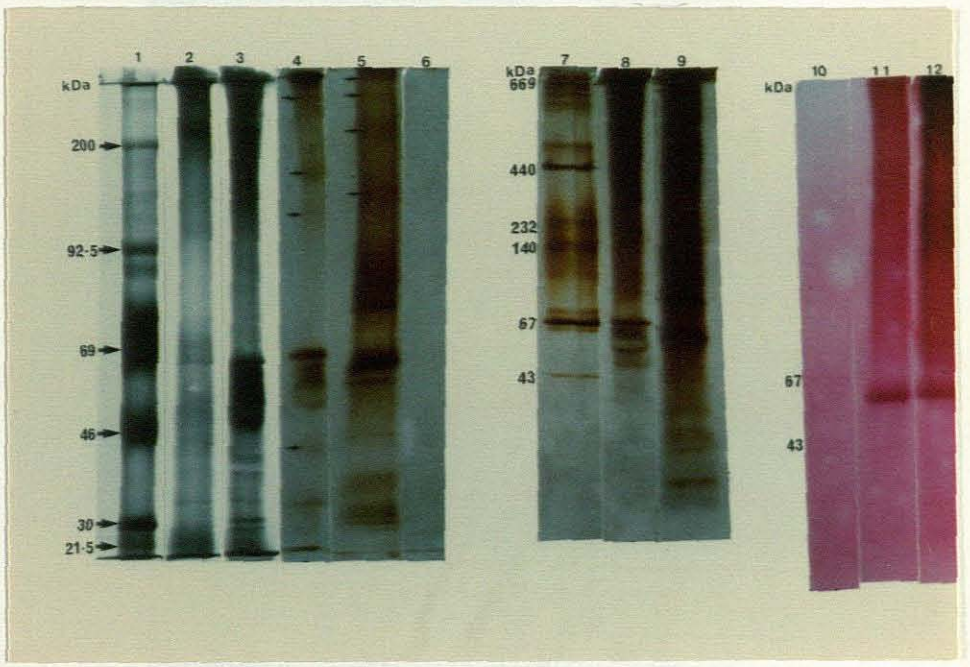



Plate 2

0.1% SDS 7.5% PAGE (lanes 1-6 stained for protein)

0.1% SDS 4%-20% gradient PAGE (lanes 7-9 stained for protein)

o.1% SDS 4%-20% gradient PAGE (lanes 10-12 stained for mucin)

Lane No.

1. MW Std (x10-³)
2. HD
3. HCA
4. HCA (G-50, peak 1)
5. HCA (G-50, peak 1 with MeSH)
6. HCA (G-50, peak 11)
7. MW Std (x10-³)
8. HCA (G-50, peak 1)
9. HCA (G-50, peak 1 with MeSH)
10. MW Std (x10-³)
11. HCA
12. HCA

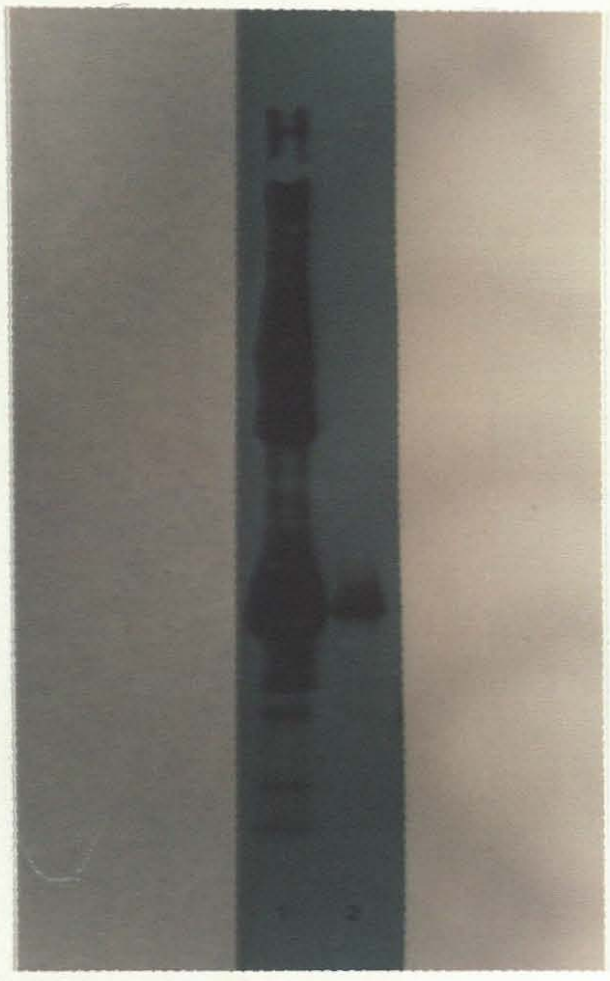



Plate 3

Western Blot

Lane No.

1. HSA (human serum albumin)

2. HCA

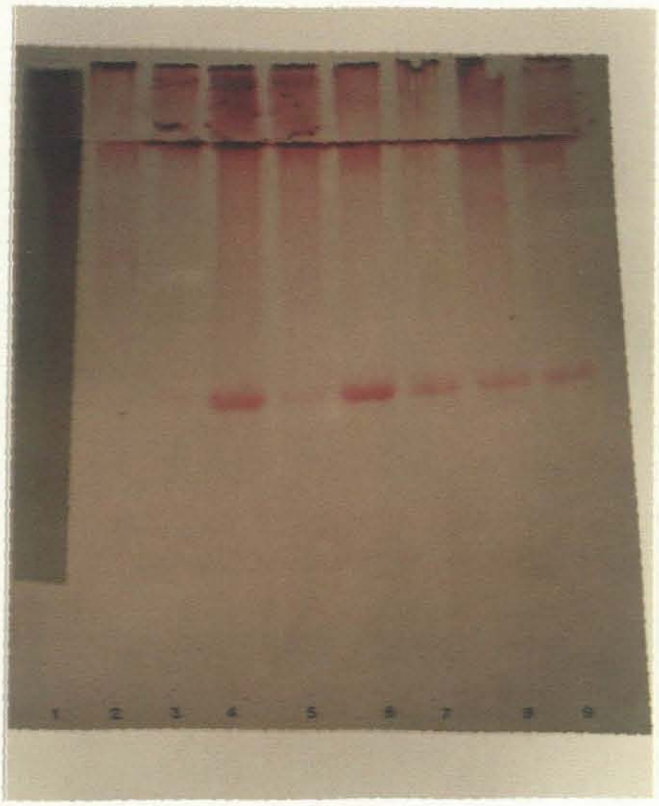


Plate 4

0.1% SDS 4%-20% gradient PAGE (stained for mucin)

Lane No.

| | | |
|---|---|---|
| 1. PM | 2. HD | 3. HCA |
| 4. HCA | 5. HCA | 6. HCA |
| 7. HGU | 8. HGU | 9. HGU |

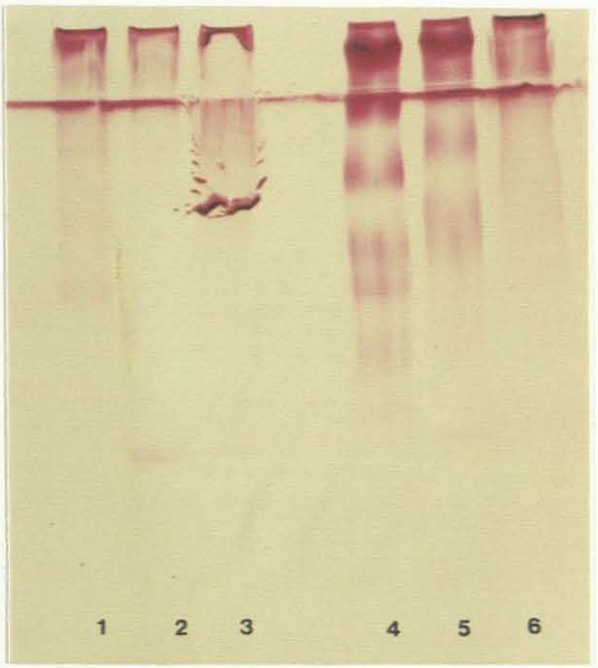



Plate 5

0.1% SDS 4%-20% gradient PAGE stained for mucin

Lane No.

1. HD
2. HGU
3. HCA
4. HD with MeSH
5. HGU with MeSH
6. HCA with MeSH

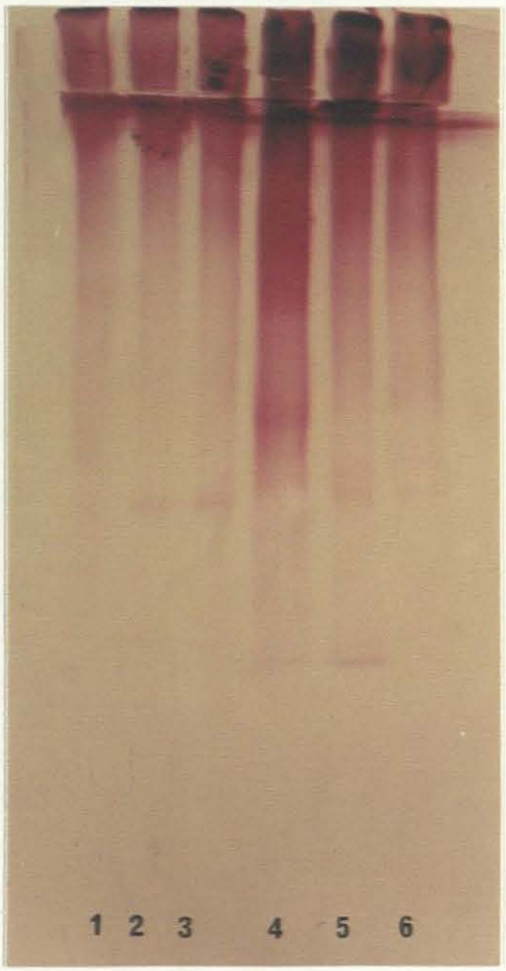



Plate 6

0.1% SDS 4%-20% gradient PAGE stained for mucin

Lane No.

1. PM
2. HCA
3. HGU
4. PM with MeSH
5. HCA with MeSH
6. HGU with MeSH

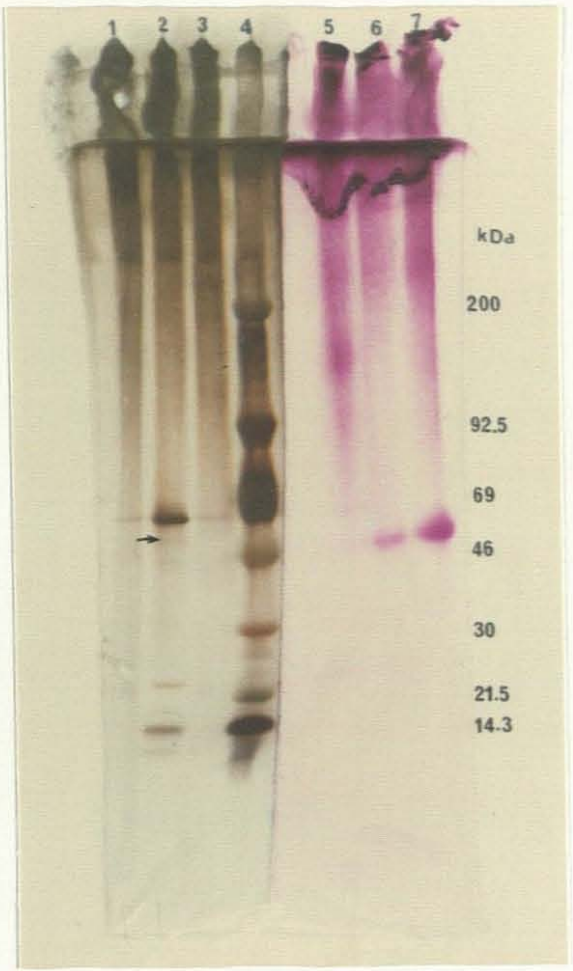



Plate 7

0.1% SDS 4%-20% gradient PAGE

(lanes 1-4 stained for protein)

(lanes 5-7 stained for mucin)

Lane No.

1. HD 2. HCA 3. HGU

4. MW Std (x10$^{-3}$)

5. HD 6. HCA 7. HGU

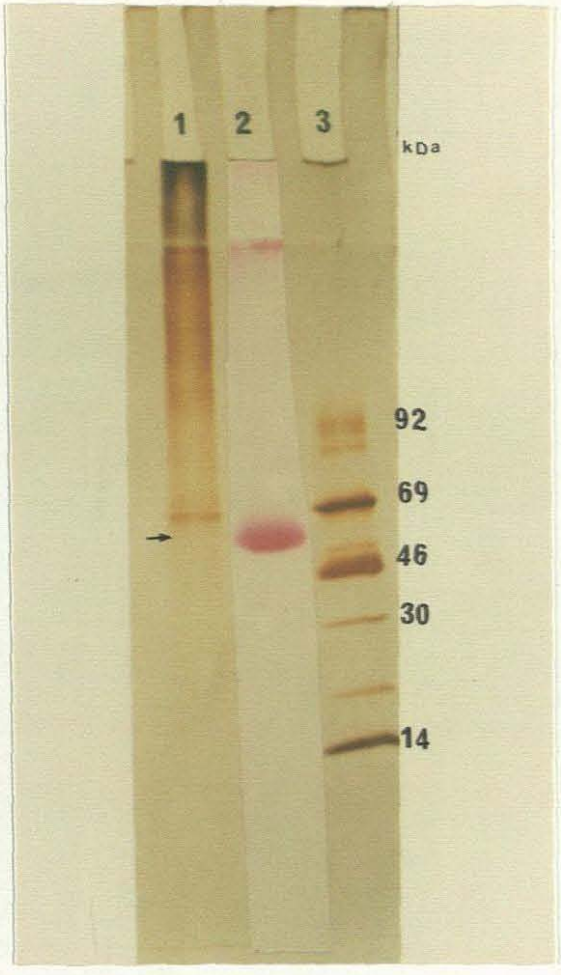



Plate 8

0.1% SDS 4%-20% gradient PAGE

(lanes 1 and 3 stained for protein)

(lane 2 stained for mucin)

Lane No.

1. HCA
2. HCA
3. Mw Std ($x10^{-3}$)


Plate 8

0.1% SDS 4%-20% gradient PAGE stained for mucin

Lane No.

1. HD pool
2. PM pool
3. HGU pool
4. HCA pool

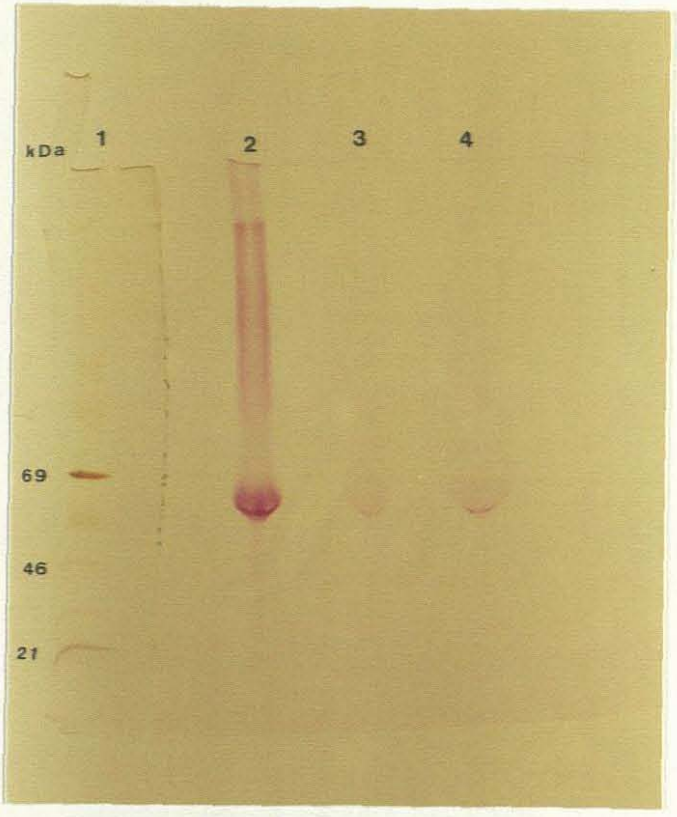



Plate 10

0.1% SDS 4%-20% gradient PAGE stained for mucin

Lane No.

1. MW Std ($x10^{-3}$)
2. 500 mcg BSA (bovine serum albumin) with <100mcg sample
3. 500 mcg BSA
4. 1000 mcg BSA

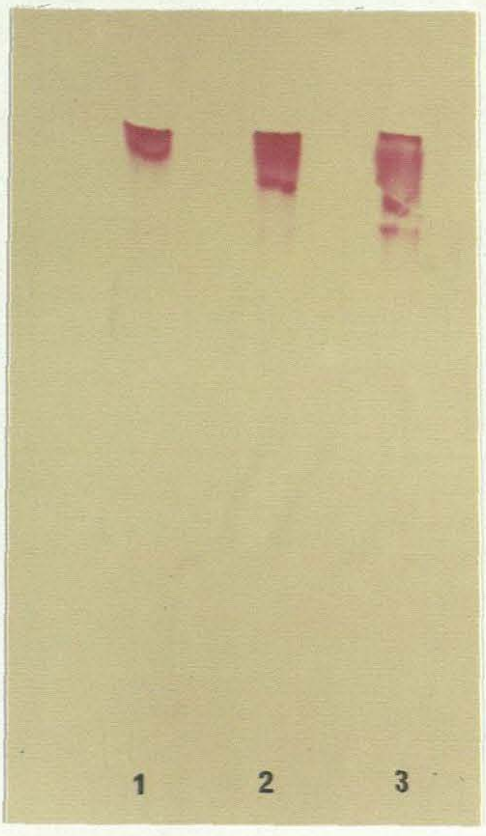



Plate 11

0.1% SDS 4%-20% gradient PAGE stained for mucin

Lane No.

1. PM
2. HGU
3. HCA

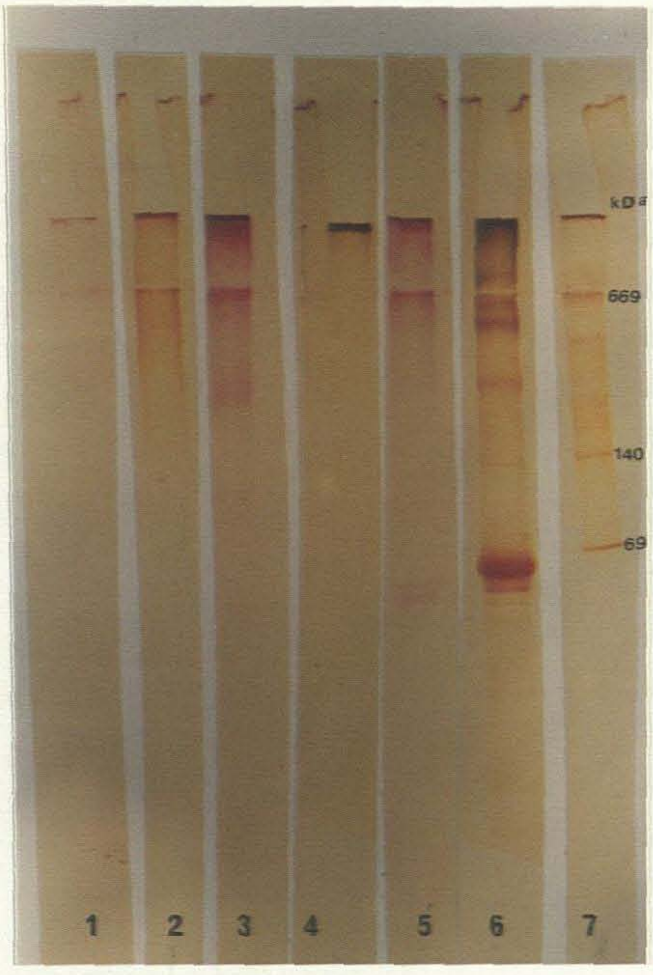



Plate 12

0.1% SDS 4%-20% gradient PAGE

(lanes 2, 4, 6 and 7 stained for protein)

(lanes 1, 3 and 5 stained for mucin)

Lane No.

1. PM (mucin)
2. PM (protein)
3. HGU (mucin)
4. HGU (protein)
5. HCA (mucin)
6. HCA (protein)
7. MW Std ($x10^{-3}$)

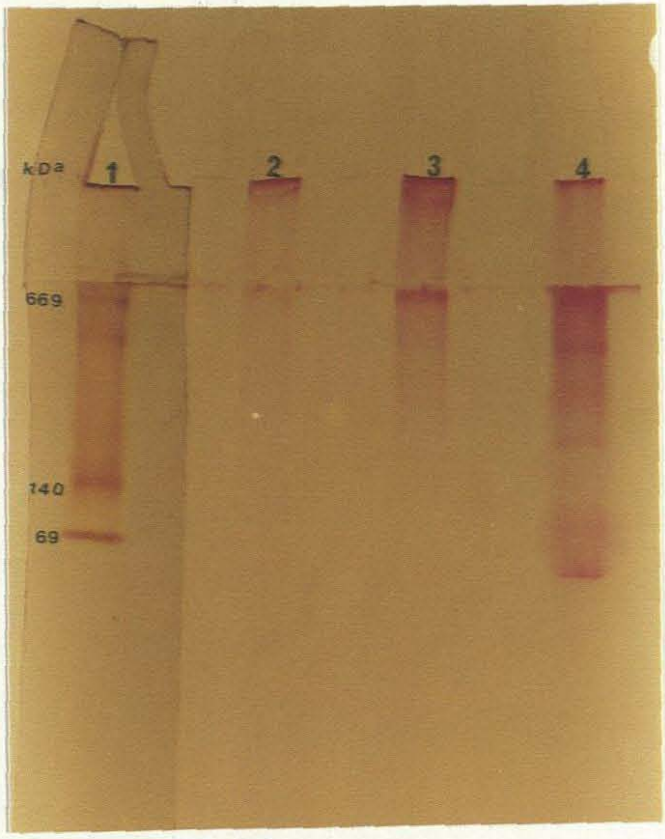



Plate 13

0.1% SDS 4%-20% gradient PAGE

(lane 1 stained for protein)

(lanes 2, 3 and 4 stained for mucin)

Lane No.

1. MW Std (x10-3)
2. HCA gastric juice (Seph. 2B $V_o$)
3. HCA gastric juice (Seph. 2B $V_i$)
4. HCA gastric juice (Seph. 2B $V_t$)

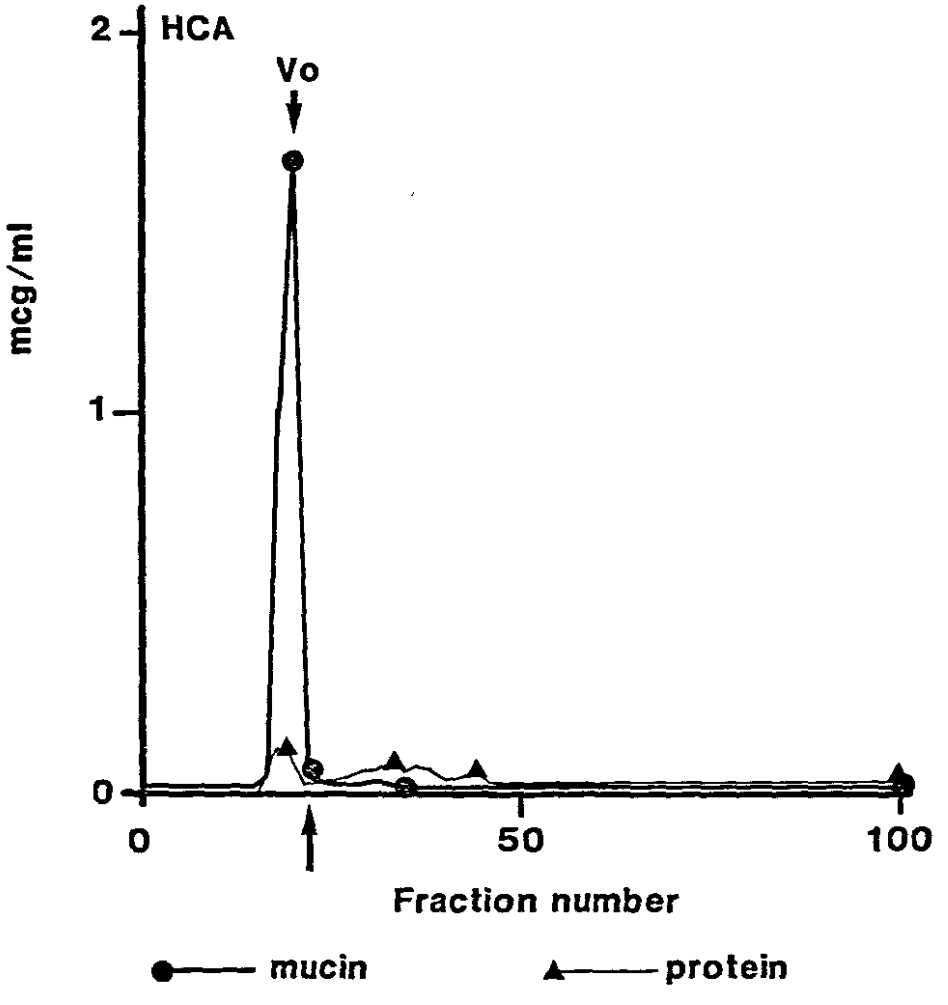



Figure 16

Purified human cancer mucin was reconstituted in 0.2M NaCl:0.02% sodium azide and chromatographed on a Sephadex G-50 column. The eluted fractions were assayed for glycoprotein (PAS) and protein (Lowry).

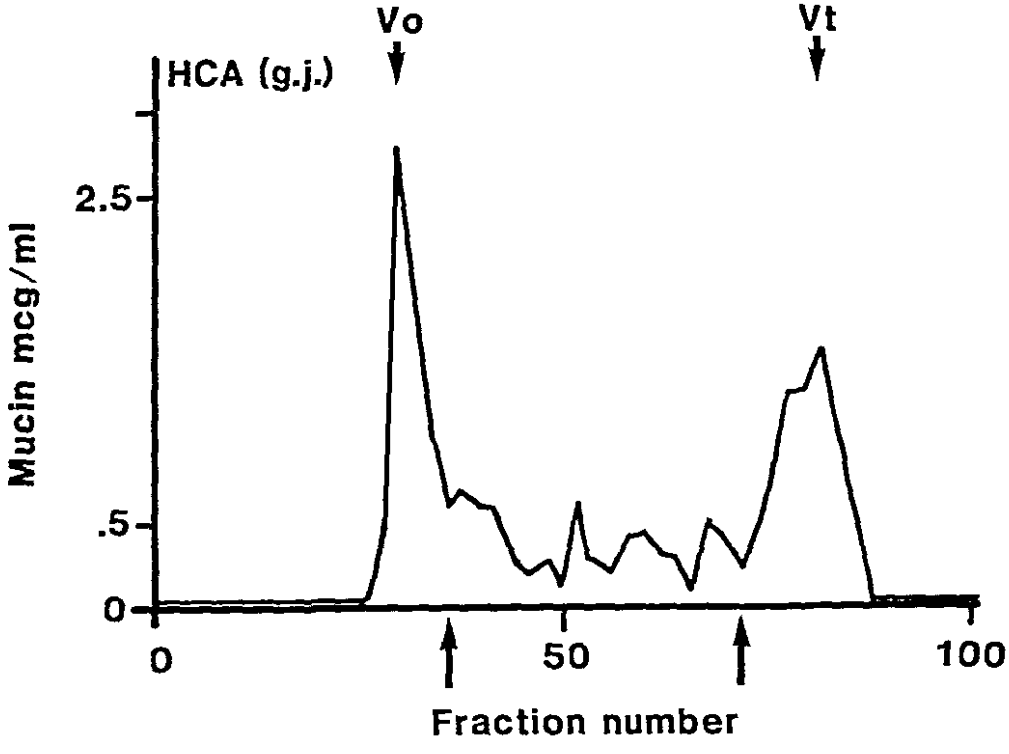



Figure 17

Partially purified gastric juice was applied to a Sepharose 2B gel filtration column and eluted with 0.2M NaCl:0.02% sodium azide (w/v). The $V_o$, $V_i$ and $V_t$ volumes were seperately pooled, dialysed against distilled water and freeze dried.

# CHAPTER 5.

# CONCLUSIONS

# 5. CONCLUSIONS

Gastric mucins were successfully isolated from mucus secretions in normal (transplant donor and post-mortem) and diseased (ulcer and cancer) states by caesium chloride density gradient ultracentrifugation. Stomachs obtained from cadavers yielded larger quantities of scrapable material off the mucosal surface than those in the other groups. Mucins of all groups were extensively degraded and displayed heterogeneity in size as shown by both Sepharose 2B and 4B gel filtration and SDS-polyacrylamide gel electrophoresis. The degradation of these mucins was of the order HCA> HGU> PM> HD. SDS-polyacrylamide gel electrophoresis, followed by staining for carbohydrate with PAS, showed that mucins of ulcerated and cancerous stomachs cofractionated in the caesium chloride gradient with a glycosylated fragment of $M_w$55-65kDa. Staining of gels for protein with $AgNO_3$ showed the fragment to be associated with a distinct band at $M_w$68kDa; Western blot analysis, using an anti-human albumin antibody revealed the $AgNO_3$ positive protein band to be albumin. Differential staining of gels with $AgNO_3$ and PAS not only confirmed the association of albumin with the glycosylated fragment but also eliminated any possibility of artefactual staining of this protein with PAS. This was further evidenced when trace amounts of the glycoslated factor mixed with albumin reacted strongly with PAS, while much larger amounts of albumin alone were hardly visible Although the albumin-glycosylated factor complex was non-

dissociable from the mucins in a 3.5M caesium chloride density gradient, dissociation could successfully be achieved by heat treatment of the mucin in SDS. The albumin-glycosylated factor complex eluted on a Sepharose 2B column as a distinct protein positive peak near the total volume of the column. The complex was either absent or present in trace quantities in post-mortem and transplant donor mucins. Preliminary studies showed this complex to be present in gastric juice of a patient with gastric cancer.

# CHAPTER 6.

# DISCUSSION

# 6. DISCUSSION

Gastric cancer is endemic in the Coloured population of the Western Cape region of South Africa. This study was undertaken with the aim of eventually developing a diagnostic marker for the detection of premalignant gastric disease in this high risk group in the Cape Town area of South Africa.

To our knowledge, the first attempt to characterise mucins in gastric carcinoma with the intention of identifying a marker for pre-malignant disease, was reported by Bara et al, (1978). A sulphoglycopeptide was isolated from these secretions to which a polyclonal antibody was developed. Cross reaction of this antibody with other mucin secretions in the gastrointestinal tract limited its use as a marker for premalignant gastric disease. The lack of sophisticated techniques to isolate and purify mucins from homogenised gastric tissue as opposed to mucosal scrapings (as done in this study) would increase the limitations described above. Later studies (Feizi et al, 1984) described altered glycosylated patterns for mucins in gastric carcinoma. Thus far many antigens have been shown to be present in the oligosaccharide side-chains of mucins e.g. blood group antigens (ABO and Lewis types), stage specific embryonic antigen (SSEA) and Forssman antigen. The purpose of our study was to find a reproducibly occurring "signature" mucin isolated from mucus secretions of cancerous stomachs using the technique shown to be the best for mucin purification,

namely, caesium chloride density gradient ultracentrifugation. It has been shown that both the composition and structural configuration of mucins have antigenicity (Mantle et al, 1984) and the approach used in our study encompassed this consideration. Finally, Price et al (1990), have reported the development of a murine anti-(human gastric carcinoma) monoclonal antibody, GL-013 that binds to a threonine rich peptide epitope expressed within the protein core of gastrointestinal mucins; its value as a diagnostic marker for premalignant disease is at present under investigation.

Gastric mucus research all over the world has long been hampered by the difficulty of obtaining mucus gel off the mucosal surface from normal subjects for use as controls in any study. This particular study posed the same problem. Although the use of human donor mucus scrapings had the advantage of being fresher, having been available much sooner than post-mortem specimens, this study showed them to be as degraded as the post-mortem mucins. The possible reasons for this have been discussed in Chapter 4 sections 2 and 3.

An alternative to this approach would be to study mucins in gastric juice from normal and diseased subjects. It has been mentioned that these mucins are not entirely degraded as has been claimed by previous researchers (Allen, 1981). This study has also shown that the albumin associated glycosylated factor of $M_w$-65kDa is present in the juice of gastric cancer

patients. The development of a protocol in which gastric juice is more thoroughly investigated would be simpler since the juice can easily be obtained by oro-gastric intubation of normal and diseased subjects. Also added to this protocol would be a separate group of samples (comprising both mucus gel and gastric juice) from normal and diseased subjects; in this instance mucins would be extracted in the presence of guanidinium chloride and a cocktail of proteolytic inhibitors to rule out the slightest possibility of *in vitro* degradation. Our intention of having a closer look at possible differences in mucin polymers in the various groups, could be done since some polymer has been shown to be present in human (this study) and pig gastric juice (Mall, unpublished).

Another exciting possibility would be to culture gastric mucous cells. Very recently Lewin of France, (1991) reported the establishment of an HGTI cell line from the fundus region of the stomach of a patient with gastric cancer. These workers showed these cells to be viable in culture with parietal cells expressing receptors for acid secretory processes. Such a culture would provide a lever for the study of both secreted and intracellular (newly synthesised and stored) mucins. An investigation into the effects of various agents on these cellular mechanisms could then also be undertaken. A specific secretion, such as the one reported in this study could then be harvested in larger quantities and its chemical and antigenic properties could then be

elucidated.

This study has shown that mucins in gastric carcinoma are more degraded and heterogeneous than their counterparts in ulcer disease and in postmortem and human donor specimens. A glycosylated factor, closely associated with albumin co-fractionates with mucins during their isolation and presents mainly in scrapings obtained from ulcerated and carcinomatous stomachs. Protocols are being designed to investigate the properties of this factor, the occurrence of which is novel for it never having been previously reported. Its possible use as a diagnostic marker for premalignant disease and a search for the presence of other possible markers within mucus secretions is part of an ongoing study.

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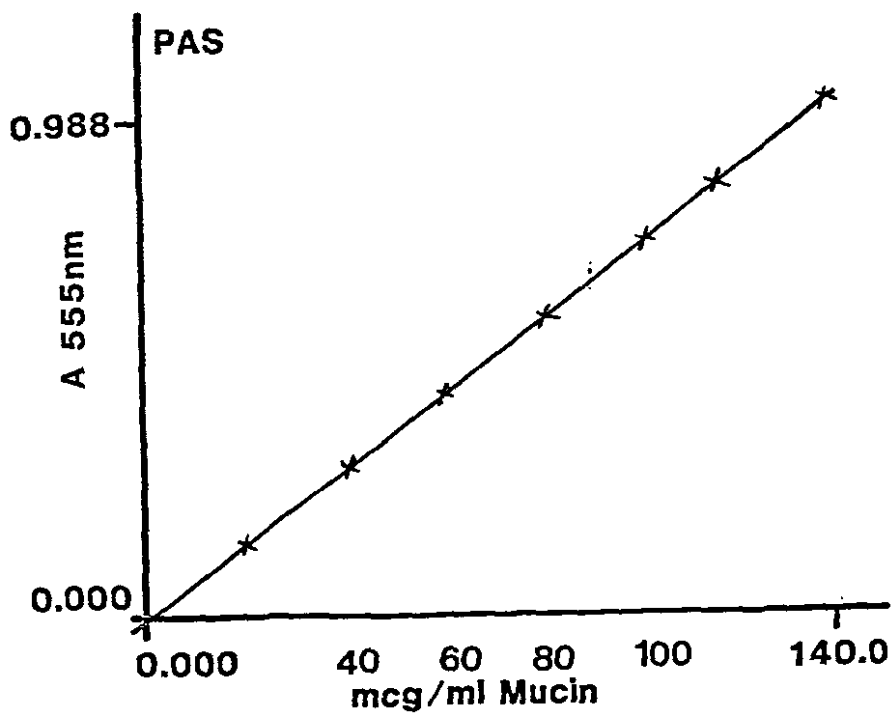
# APPENDIX A

Standard curves (20-140mcg/ml in 0.2M sodium chloride and 0.02% (w/v) sodium azide) were constructed from pig gastric mucin which had been purified twice in caesium chloride.



# APPENDIX B

Varying concentrations (20-100mcg/ml) of bovine serum albumin in 0.2M sodium chloride and 0.02% (w/v) sodium azide were used to construct a standard curve.