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REGULATION OF MUCUS SECRETION BY CELLS ISOLATED FROM THE RAT GASTRIC MUCOSA

by

Andrew Clive Keates

A thesis submitted for the degree of Doctor of Philosophy

ASTON UNIVERSITY

March 1991

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This study was undertaken to further understanding of the mechanisms which regulate mucus secretion by rat stomach cells. Particular objectives were: (i) to develop and use a radiochemical assay to estimate the secretion of mucin by a suspension of gastric mucosal cells in vitro, (ii) to develop and use a solid-phase enzyme immunoassay (EIA) to study the regulation of the release of bulk gastric mucin from the isolated cells and (iii) to compare the results obtained with the two procedures.

Cells were isolated by exposure of gastric mucosa to pronase and EDTA. Cell suspensions were preincubated with D-[6-^{3}H]glucosamine. [^{3}H]-labelled material of high molecular mass released into the incubation medium, was purified by Fast Protein Liquid Chromatography, and appeared to be gastric mucin. Some unidentified [^{3}H]-labelled material of lower molecular mass was also found in the medium. Release of [^{3}H]-labelled high molecular mass material was essentially linearly related to time. Secretin, isoprenaline and carbachol stimulated release of [^{3}H]-labelled high molecular mass material. The half-maximally effective concentrations of secretin and isoprenaline were 2.3nM and 34nM respectively. Histamine, gastrin and epidermal growth factor were without effect.

A rabbit polyclonal antibody was raised by using purified 'native' rat gastric mucin as immunogen. The antibody preparation appeared specific for rat gastric mucin and was used to establish a quantitative solid-phase EIA. Release of bulk mucin was essentially linearly related to time. Phorbol-12-myristate-13-acetate (PMA), forskolin and A23187 dose-dependently stimulated bulk mucin release. Synergistic interactions were observed between PMA and forskolin, and PMA and A23187. Secretin and isoprenaline were confirmed as mucin secretogogues.

In conclusion gastric mucin release was investigated for the first time by using a suspension of gastric mucosal cells. Two different assay procedures were developed. Some pathways and agents responsible for controlling mucin secretion were identified.

Key words: Mucin, gastric secretion, gastric mucosa, ELISA.

For my Family.

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ABBREVIATIONS

The following, non-standard abbreviations were used throughout this work.

ANOVAR	Analysis of variance
dbcAMP	dibutyryl cyclic AMP
DMSO	dimethylsulphoxide
EC_{50}	concentration of an agent producing a half-maximal stimulatory effect
EDTA	ethylenediaminetetra-acetic acid
EIA	enzyme immunoassay
ELISA	enzyme-linked immunosorbent assay
Fig	figure
FPLC	Fast protein liquid chromatography
h	hour(s)
kDa	kilodalton (dalton = 1/12th of the mass of latom of nuclide ^{12}C)
min	minute(s)
PBS	phosphate-buffered saline
PMA	phorbol-12-myristate-13-acetate
r.p.m.	revolutions per minute
s	second(s)
S.E.M.	standard error of the mean
TBS	tris-buffered saline

Chapter One

INTRODUCTION

A viscoelastic layer of gel called mucus covers the epithelial cell surface of the stomach. The gel-forming components of gastric mucus are the mucins or mucous glycoproteins. This chapter will provide a short review of the anatomy of the gastric mucosa and of the structure, function, synthesis and secretion of gastric mucin.

1.1 ANATOMY OF THE GASTRIC MUCOSA

1.1.1 Gross morphology

The mammalian stomach of most non-ruminant animals forms an expanded region of the gastrointestinal tract between the oesophagus and the duodenum. Ingested food is temporarily stored in this sac whilst being initially digested. The rat stomach can be broadly divided into two regions. The oesophagus opens into a non-glandular area called the forestomach or cardiac region. This area is only involved in food storage and is covered by a keratinized stratified squamous epithelium. The major part of the stomach is the fundus. This region of the stomach is glandular and contains numerous tubular invaginations, the gastric glands, from which acid (which may help to sterilize the food) and pepsinogen (which is converted to pepsin at low pH) are secreted (Fig 1.1). In the rat an antral glandular region also exists between the fundus and duodenum, which can be identified by the anatomy of its characteristic pyloric glands.

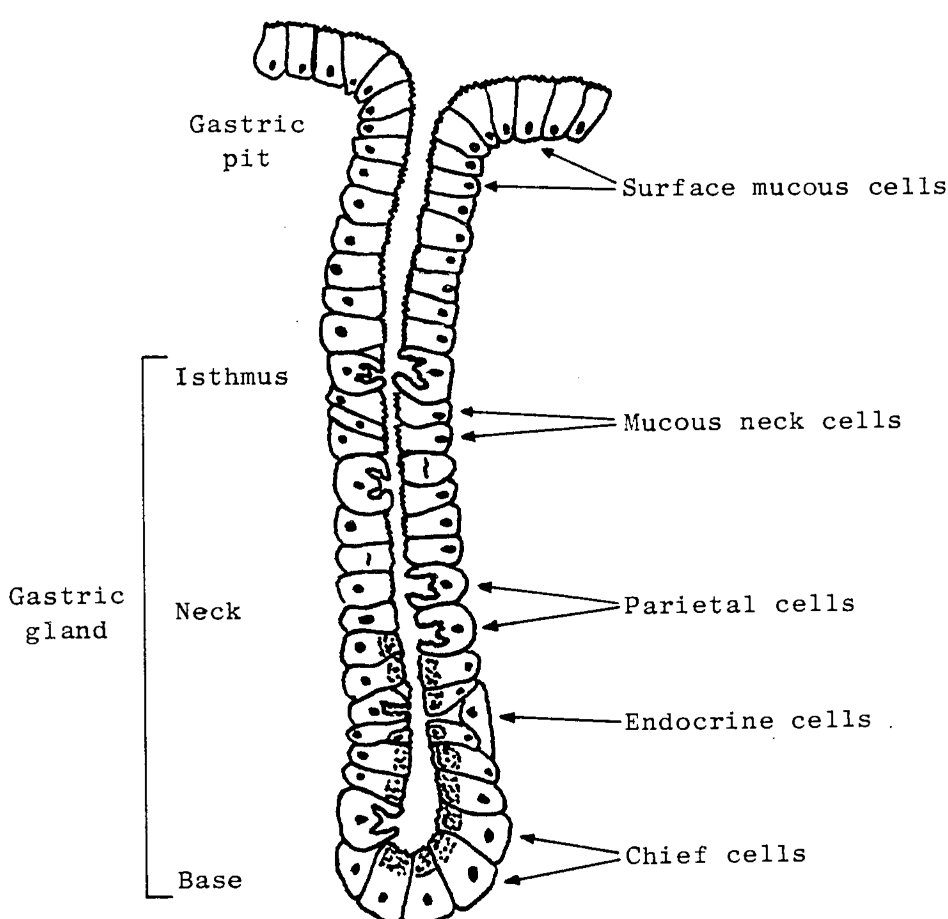
1.1.2 Cell-types of the mucosa

1.1.2.1 The mucous cells

Two types of cell which contain mucin are present in the mammalian stomach: (i) surface mucous cells and (ii) mucous neck cells.

Figure 1.1

A schematic diagram of a gastric gland from a mammalian stomach.

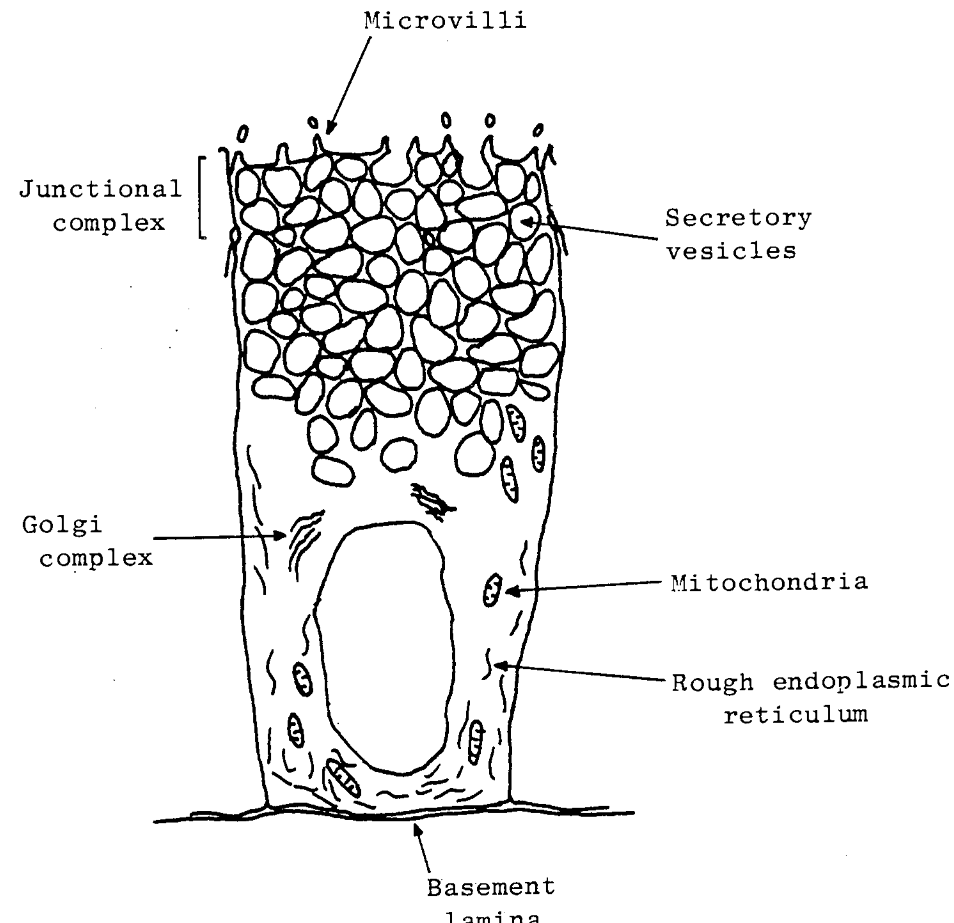


The surface mucous cells (Fig 1.2) cover the luminal surface of the glandular epithelium and also extend into the pit and isthmus of the glands. These cells are a simple columnar epithelium joined to each other via belt-like tight junctions (Farquhar & Palade, 1963). These barriers prevent the passage of large molecules across the epithelial sheet and restrict the exchange of apical membrane components with those in the basolateral membrane. The intracellular organisation of the surface mucous cell is typical of exocrine secretory cells. The rough endoplasmic reticulum, golgi complex, mitochondria and nucleus are usually concentrated in the basal and lateral cytoplasm, whilst secretory granules containing mucin are stored in the apical cytoplasm. Indeed stains specific for carbohydrate, such as the periodic acid/Schiff (PAS) reaction intensely stain the apical cytoplasm of surface mucous cells. Surface mucous cells are continuously lost from the luminal surface of the epithelium, either through cellular degeneration or mechanical sloughing into the lumen (Lee & Leblond, 1985; Tatsumi et al., 1985). Cells lost in this manner are continually replaced by proliferation of multipotent endodermal stem cells which are capable of differentiating into all the specialized cell-types present in the epithelium (Williams, 1977). The process of proliferation and migration takes approximately three days (Hunt & Hunt, 1963).

The gastric mucosa also contains mucous neck cells in addition to surface mucous cells. These cells are present in the neck and isthmus region of glands where a gradual transition from surface to neck cells occurs. Mucous neck cells appear to be four.' in close proximity to parietal cells (1.1.2.2). The cytoplasm of the mucous neck cells is essentially similar to that of the surface mucous cells. Mucous neck cells exhibit apical staining with PAS which is similar to mucous epithelial cells, but, staining with alcian blue at low pH is more intense for mucous neck than for epithelial cells

Figure 1.2

A schematic diagram of a surface mucous cell.



(Spicer et al., 1978). The staining pattern obtained with alcian blue may be a consequence of the presence of a greater proportion of sulphated (acidic) mucin in mucous neck cells than in mucous epithelial cells. Thus, uptake of radioactive sulphur has been demonstrated in dog mucous neck cells (Zalewsky & Moody, 1979). Mucous neck cells are lost and replaced similarly to surface mucous cells, however renewal usually requires about one week (Hunt & Hunt, 1963).

1.1.2.2 Other cell-types

The other major cell-types present in the gastric mucosa are the acid-secreting (parietal) cells, the pepsinogen-secreting (chief) cells and endocrine cells. Some features of these cell-types are summarized in Table 1.1.

1.2 GASTRIC MUCUS

1.2.1 Localization and composition of gastric mucus

1.2.1.1 Phases of gastric mucus

Gastric mucus exists in three distinct forms in the stomach (Allen et al., 1989): (i) a water-insoluble gel layer adherent to the mucosal surface, (ii) soluble mucus present in the luminal contents, and (iii) presecreted intracellular mucus stored in apical secretory granules. The mucous gel layer is involved in protecting the gastric epithelium from acid and peptic attack (1.2.3). In unfixed sections of gastric mucosa this layer has been observed to be a continuous barrier of 80µm (rat) and 180µm (human) mean thicknes (Kerss et al., 1982). Fixation of tissue in situ using anti-mucus antibodies (Bollard et al., 1986) or freeze-vapour substitution (Sakata & Englehart, 1981) also reveals an uninterrupted gel layer in electron micrographs and histological sections respectively.

Table 1.1

Features of some other cell-types present in the gastric mucosa

Cell-type	Location	Secretion

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peptide

* The cells listed above are the major types present in the gastric mucosa. Up to nine separate endocrine cells have been identified in the gastrointestinal tract (Grube & Forssmann, 1979).

Soluble mucus present in the luminal contents of the stomach is a degradation product of the insoluble mucus gel layer. This form of mucus is generated by a combination of mechanical abrasion and the proteolytic activity of pepsin (1.2.2.2). Soluble mucus may act as a lubricant during digestion. The third form of mucus is that stored in the secretory vesicles. The mucin present in these granules is highly condensed.

1.2.1.2 Composition of mucus gel

The main component of the mucus gel layer is water which can be upto 95% by weight, however the most abundant structural component is mucin. It is now generally accepted that the physical properties of undegraded mucins are responsible for the gel-forming nature of mucus. (Bell et al., 1984; Sellers et al., 1988). Thus, purified gastric mucin at physiological concentrations (approximately 50mg/ml) can be reconstituted into a gel with identical rheological properties to native mucus. The structural features of gastric mucin are presented in 1.2.2.

Minor components of mucus are cellular proteins, nucleic acids and lipids most of which are probably derived from sloughed epithelial cells (Forstner, 1978). Bacteria e.g. Helicobacter pylori may also be present.

In addition to the common cellular lipids found in the mucus gel, uncommon disaturated phosphatidylcholine, phosphatidylethanolamine and sphingomyelin phospholipids containing two palmitoyl acyl groups have also been reported (Wassef et al., 1978, Lichtenberger et al., 1982). This class of phospholipids has only been previously demonstrated at the air-mucus interface in lung surfactant (Hills, 1982) where surface hydrophobicity is increased by the formation of a lipid monolayer. The significance of

these molecules in the mucus gel is poorly understood, but they may make this layer a hydrophobic barrier to the hydrophilic luminal contents.

1.2.2 Structure of gastric mucin (mucous glycoprotein)

1.2.2.1 Composition

Mucin-type glycoproteins have a distinctive composition which differs significantly from that of serum glycoproteins, which predominantly contain asparagine-linked carbohydrate, and proteoglycans which contain D-glucuronic acid and L-iduronic acid residues (Allen, 1983).

The protein component of mucins, which exists as a thread-like central polypeptide core is only 15-20% by weight of the molecule (Scawen & Allen, 1977; Pearson et al., 1981), with serine, threonine and proline accounting for 40-50% of the amino acid content. Carbohydrate residues (fucose, galactose, N-acetylglucosamine, N-acetylgalactosamine and sialic acids) represent approximately 80% by weight of the molecule. The sugar side-chains are heterogenous varying between 4-19 residues in length (Allen, 1983; Slomiany et al., 1984a,b) and are attached to the polypeptide component in distinct areas of glycosylation by O-glycosidic bonds between N-acetylgalactosamine residues and the hydroxyl groups of serine and threonine residues. These areas of the polypeptide core also contain large amounts of proline, which is presumably required to achieve a conformation necessary for the close packing of the carbohydrate side chains. (Allen, 1983). A further feature of mucin molecules is the presence of small amounts of sialic acid and ester sulphate residues (Allen et al., 1984b; Allen & Snary, 1972; Scawen & Allen, 1977) which gives these molecules an overall negative charge.

In addition to the monosaccharide composition listed previously, small amounts of mannose have been detected in rat gastric mucin (Dekker et al., 1989b), which is likely to have originated from asparagine (N) - linked carbohydrate. These authors have also found that inhibition of N-glycosylation with tunicamycin or cleavage of N-linked glycans using endoglycosidase H reduced the molecular mass of [^{35}S]-labelled rat gastric mucin precursors, isolated by immunoprecipitation, from 300 kDa to 270 kDa (Dekker et al., 1989b), implying the presence of N-linked glycans.

1.2.2.2 Overall polymeric structure

The molecular mass (kDa) of isolated undegraded gastric mucin molecules is controversial and depends upon the extraction and purification procedure employed (Allen et al., 1984a; Carlstedt & Sheehan, 1984a). Hence analysis of pig gastric mucin isolated by gentle stirring in the presence of the chaotropic agent guanidine hydrochloride by sedimentation velocity gave a $S^0_{20,w}$ value of 77S and an approximate molecular mass of 44000kDa (Carlstedt & Sheehan, 1984a). In contrast sedimentation analysis of pig gastric mucin extracted by homogenization in 3.5M-caesium chloride without protease inhibitors gave a single, unimodal, polydisperse peak with a $S^0_{25,w}$ value of 33S and an approximate molecular mass of 2000 kDa (Hutton et al., 1983). Furthermore these authors showed that pig gastric mucin isolated in the presence of guanidine hydrochloride and/or protease inhibitors gave two major peaks on sedimentation analysis with $S^0_{25,w}$ values of 33S and 42S-110S. Treatment of the heterogeneous material with 1% (w/v) sodium dodecyl sulphate at 100^{0}C for 10 min gave a single, polydisperse peak with a $S^0_{25,w}$ value of 33S-265, indicating that mucin isolated using guanidine hydrochloride probably gives rise to non-covalent aggregates.

Further physicochemical data for gastric mucin such as light scattering (Carlstedt & Sheehan, 1984a) suggest that these molecules are highly expanded, take on a spheroidal solvent domain and have a behaviour approaching that of a random coil. This view is further supported by electron microscopy which suggest that isolated gastric mucins are elongated thread-like structures, (Dekker et al., 1989a; Hutton et al., 1988), with no evidence of branching of the mucin chain. A schematic representation (not to scale) of undegraded gastric mucin is presented in Fig 1.3.

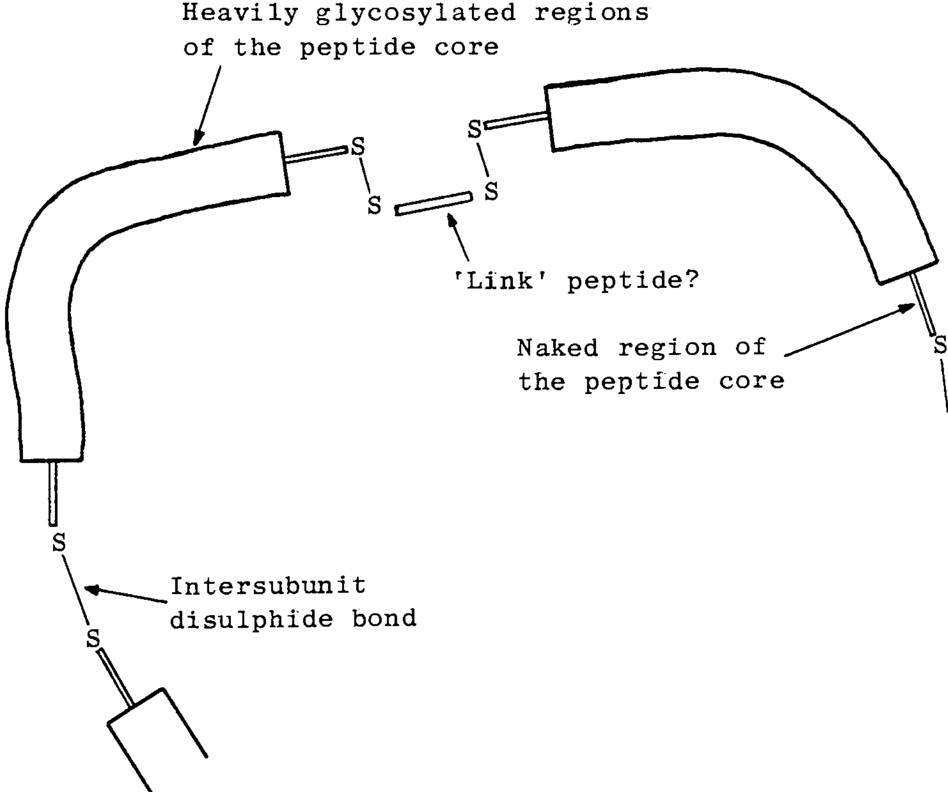
Gel formation by mucin polymers is thought to result from stable non-covalent intra/intermolecular interdigitations between the carbohydrate side-chains present on mucin molecules (Sheehan & Carlstedt, 1984; Bell et al., 1985; Sellers et al., 1988). Clearly mucin concentration is therefore of prime importance in gel formation, since it governs the degree of side-chain interaction.

1.2.2.3 Mucin 'subunits' and 'glycopeptides'

The functional configuration of undegraded gastric mucin is a disulphide linked polymer (Allen, 1983). Reduction of the disulphide bonds with thiol agents such as 2-mercaptoethanol yield soluble mucin 'subunits' and the viscoelastic properties of the gel are abolished (Bell et al., 1985; Pearson et al., 1981). Two different molecular masses of subunit have been reported depending on the isolation method employed (Allen et al., 1978; Carstedt et al., 1983). Reduction of pig gastric mucin extracted into a solution containing protease inhibitors with/without 6M-guanidine hydrochloride followed by two or three caesium chloride density gradient centrifugation steps with 10mM-dithiothreitol gave subunit masses of approximately 2000 kDa and 500kDa respectively by sedimentation analysis. Further treatment of the high molecular mass 'subunit' with 0.2M-2-mercaptoethanol

Figure 1.3

A schematic representation of gastric mucin.



reduced the molecular mass to 500kDa (Mall et al., 1988). The higher molecular mass 'subunit' has been proposed to represent a stable intermediate between that of the polymer and subunit.

The action of proteases such as trypsin, pepsin, pronase and papain upon the structure of undegraded gastric mucin has been well-studied (Scawen & Allen, 1977; Pearson et al., 1980; Bell et al., 1985). Proteolytic degradation of mucin polymers is achieved by cleavage of the central protein core in the non-glycosylated regions (Fig 1.3) yielding soluble mucin 'glycopeptides' resistant to further proteolysis. The molecular mass of such subunits has been estimated to be approximately 500kDa (Scawen & Allen, 1977; Pearson et al., 1980).

Dekker et al., (1989b) have investigated the biosynthesis of rat gastric mucin using an antiserum specific for peptide epitopes and in pulse-chase experiments with [^{35}S]-methionine a protein of 300kDa could be immunoprecipitated from stomach segments. This protein may represent the peptide backbone in rat gastric mucin subunits, however assuming that this component only represents approximately 20% by weight of the fully mature subunit a total molecular mass of 1500kDa for rat gastric mucin subunits can be calculated. This value differs somewhat from the values obtained from reduction of pig gastric mucin with thiol agents and may represent a species difference. However, not until the gene(s) encoding rat gastric mucin are cloned and sequenced will the molecular mass of the subunit(s) be known accurately.

1.2.2.4 'Link' peptides

The precise nature of the bonding arrangements effecting the assembly gastric mucin subunits into polymers is poorly understood. A protein of 70kDa can be released from purified pig gastric mucin after

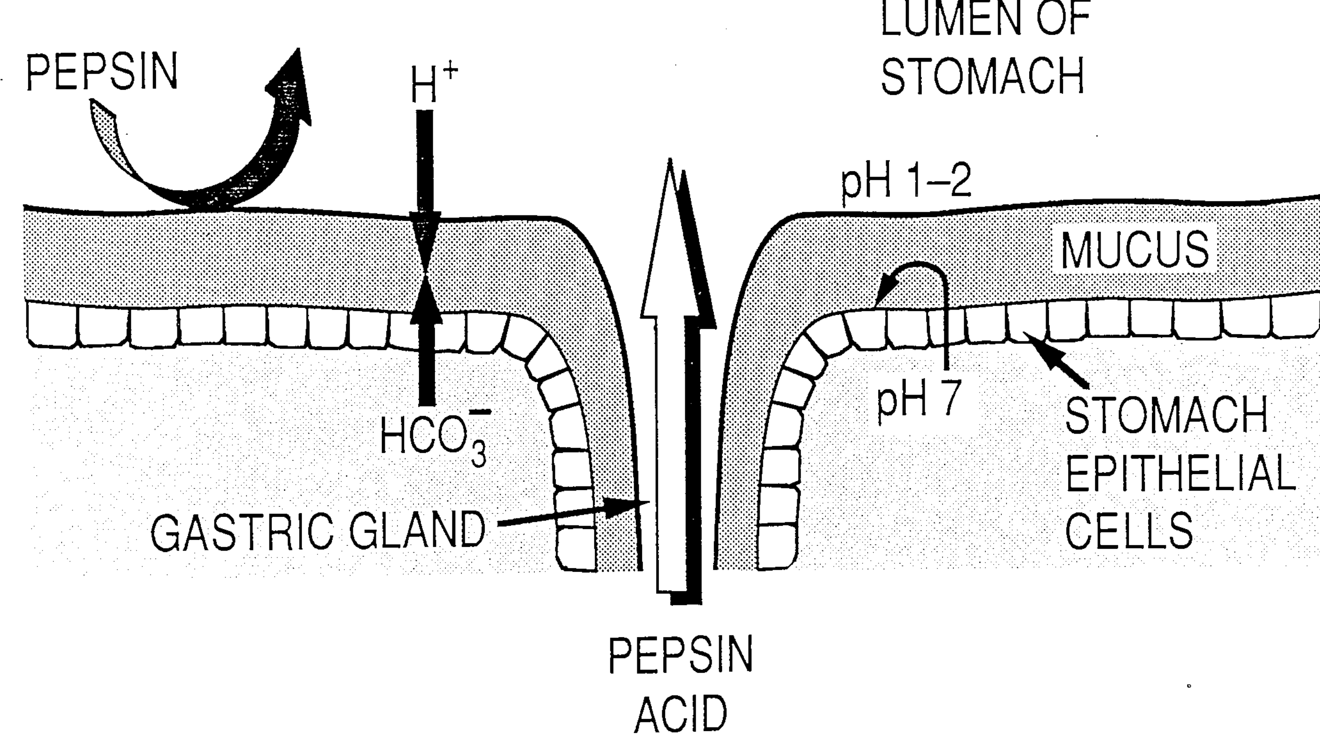
reduction of disulphide bonds with 0.2M-2-mercaptoethanol. (Pearson et al., 1981). This protein was cysteine rich and has been ascribed a function in linking mucin subunits. A 118kDa component can similarly be released from rat small intestine mucin (Mantle et al., 1984). By contrast, human cervical mucins isolated in the presence of guanidine hydrochloride do not release a 'link' peptide upon disulphide reduction (Carlstedt & Sheehan, 1984b). Gastric 'link' peptide could be a consequence of partial proteolysis of the mucin core peptide during purification, with 'nicked' fragments being released on reduction of disulphide bridges, although recent results do not support such an explanation for the origin of the small intestinal 'link' peptide (Robertson et al., 1989).

1.2.3 Functions of gastric mucus

Gastric mucus has four proposed protective functions in the stomach. Firstly, as mentioned previously soluble mucin helps lubricate the digestive process. Secondly, the adherent mucus gel acts as a physical barrier to pepsin impeding its access to the epithelium (Allen et al., 1984b). Thirdly, the adherent gel layer acts as a mixing barrier, in which acid diffusing from the lumen is neutralized by bicarbonate secreted by the epithelium (Flemstrom & Garner, 1982). The resulting pH gradient keeps the pH at the epithelial cell surface close to neutrality except when the luminal pH drops below 1.4 (Ross et al., 1981; Wallace, 1989). The second and third functions are sometimes jointly described as the mucus-bicarbonate barrier (Fig 1.1). Finally mucus in conjunction with exfoilated cells and fibrin forms a protective cap over areas of acute cellular damage. This structure facilitates regeneration of the epithelium by allowing cell migration from the gastric glands to restore epithelial continuity in a protected environment (Ito & Lacy, 1985).

Figure 1.4

Mucus-bicarbonate barrier.



1.3 BIOSYNTHESIS OF GASTRIC MUCIN

1.3.1 Synthesis of the central peptide core and addition of oligosaccharide side chains

The cells that synthesize and secrete mucin are highly polarized and show adaptations typical of exocrine secretory cells. In particular the rough endoplasmic reticulum (RER) and Golgi complex are well developed, consistent with the high synthetic activity required for mucin production.

The central peptide core of mucin is synthesized on polyribosomes present on the cytoplasmic face of the RER. The number and nature of the genes coding for the mucin protein backbone are largely univestigated, however recently two separate proteins of approximately 300 kDa molecular mass have been immunoprecipitated from rat gastric mucosa. (Dekker et al., 1989b). During synthesis the nascent peptide must be translocated across the RER membrane into the lumen of the endoplasmic reticulum, where a signal peptide is probably removed. N-glycosylation of the peptide is initiated during the translocation process with the transfer of an activated 'high mannose' oligosaccharide (Glc_3 Man_9 $GlcNAc_2$) via the lipid dolichol to asparagine residues in the amino acid sequences asparagine-x-serine or asparagine-x-threonine where x is any amino acid except proline or aspartic acid (Marshall, 1972). After peptide synthesis, translocation and initial N-glycosylation events the immature mucin is transported to the Golgi complex via vesicles and further glycosylation now occurs from UDP-N-acetylgalactosamine.

Addition of N-acetylgalactosamine to the hydroxyl groups of threonine and serine residues present in the mucin protein backbone probably occurs in the cis Golgi cisternae and marks the start of O-linked glycosylation (Deschuyteneer et al., 1988). This addition is

catalysed by a specific membrane bound glycosyl transferase which appears to require the presence of one or more proline residues in the vicinity of the amino acid acceptors (Briand *et al.*, 1981; Hanover *et al.*, 1980). Further O-glycosylation proceeds by the stepwise addition of monosaccharides (from their nucleotide activated intermediates) via specific glycosyltransferases to the initially added N-acetylgalactosamine residues (for review see Schachter and Williams, 1982).

In the Golgi complex N-linked glycans are processed from 'high mannose' to 'complex' N-linked glycans. This conversion is achieved by the removal of all of the glucose, six mannose residues and the addition of a N-acetylglucosamine residue. Similarly to O-glycosylation, N-glycosylation now proceeds by the sequential addition of monosaccharides via specific glycosyltransferases in the trans-cisternae of the Golgi complex (see Kornfeld & Kornfeld, 1985 for review).

The final stages in mucin biosynthesis is the addition of sialic acid and sulphate residues which are added in the trans-cisternae of the Golgi complex (Roth *et al.*, 1984; Kramer *et al.*, 1978).

The O- and N- linked oligosaccharide side chains present on fully processed mucin molecules display considerable heterogeneity. Such heterogeneity probably arises as a consequence of the operation of the following mechanisms: (i) determination of monosaccharide addition to growing chains by the nature of the previously attached residue and the expression of specific glycosyltransferases (ii) chain branching; (iii) competition between glycosyltransferases for common substrates; (iv) addition of monosaccharides which prevent elongation.

1.3.2 Storage of synthesized mucin

After the completion of biosynthesis and prior to their secretion into the lumen, mature polymeric mucin molecules are packaged into secretory vesicles. These structures appear to bud from the trans-Golgi cisternae. The calcium content of secretory vesicles is high (Warner & Coleman 1975) and divalent cations have been shown to reduce mucin volume in vitro (Forstner & Forstner, 1977). Therefore the role of calcium in secretory vesicles would appear to be one of packaging the stored mucin molecules, by neutralizing the negative charge on the glycoprotein.

1.4 SECRETION OF GASTRIC MUCIN

1.4.1 Mechanisms of secretion

Secretion of gastric mucin stored in secretory vesicles can be achieved via three distinct mechanisms: (i) slow release by exocytosis, (ii) rapid apical expulsion or (iii) cell exfoliation.

1.4.1.1 Exocytosis

The process of exocytosis is a poorly understood phenomenon, which involves the fusion of secretory vesicles with the apical plasma membrane and the subsequent ejection of the concentrated stored mucin into the gastric lumen. In unstimulated chambered canine gastric mucosa, excytosis involved the fusion of a few vesicles at a time and was found in 50-75% of foveolar and interfoveolar mucus cells examined by transmission micrographs. (Zalewsky & Moody, 1979), providing a steady, slow secretory rate. Stored concentrated mucin released into the lumen expands through rapid hydration, probably initiated by the loss of calcium ions present in the vesicle (Verdugo, 1984).

1.4.1.2 Apical expulsion

One form of rapid release of stored mucin is achieved by apical expulsion. This mechanism involves the fusion of intracellular secretory granules to form an apical pool of mucin, which then fuses *en bloc* with the apical membrane and is released into the gastric lumen. This form of mucin secretion was observed in 10-20% of unstimulated canine interfoveolar mucous cells identified in scanning electron micrographs (Zalewsky & Moody, 1979). After mucin release *in situ* cellular degeneration occurs releasing the cell contents into the lumen.

1.4.1.3 Cell exfoliation

The second form of rapid release of mucin occurs indirectly through cell exfoliation. In unstimulated mucosa, this method of release was observed in less than 1-2% of canine mucous cells (Zalewsky & Moody, 1979). At the ultrastructural level loss of basolateral attachment leads to the ejection of the entire cell into the lumen. This type of release is often seen with mucosal damaging agents such as non-steroidal anti-inflamatory drugs (Morris *et al.*, 1984).

1.4.2 Regulation of secretion

This thesis is an investigation of the release of gastric mucin from rat isolated gastric mucosal cells. This section of the introduction will therefore provide a short overview of the methodologies used to study mucin secretion, and of the agents known to stimulate its release that existed at the beginning of this study (October, 1987).

1.4.2.1 Methods for measuring mucin secretion

Methodologies developed to investigate gastric mucin/mucus secretion are listed in Table 1.2. Many of these procedures can be critisized.

Estimates of secretion in vivo involve quantitation of luminal sugars or the measurement of binding capacity of luminal material for the dye alcian blue. However such assays may reflect degradation of the mucous gel layer and/or wash-out of degraded (soluble) mucin into the gastric lumen. Direct measurement of the thickness of the mucous gel layer after administration of agents in vivo (either topically, intravenously or interperitoneally) is a reflection of changes in the dynamic equilibrium that exists between mucus secretion and degradation, and does not therefore represent mucin secretion per se. Although if degradation of the mucus layer is constant this procedure will provide an index of secretory activity.

For many experiments in vitro systems are preferable to in vivo systems since results with these systems are often less difficult to interpret and experimental manipulation is easier. Gastric explants or mucosal slices are a common in vitro preparation and offer certain advantages such as the ability to closely control the composition of the solutions bathing the tissues and a cellular physiology similar to that found in vivo. However these preparations have distinct disadvantages. In particular, such preparations contain nerve endings and endocrine cells which may modulate experimental observations. A further potential problem is the access of secretogogues, nutrients and oxygen to all parts of the tissue.

Advantages of primary gastric cell cultures (Terano et al., 1987; Yoshida et al., 1987) are similar to those of explants and slices except that the

Table 1.2

Some techniques used to study the regulation of gastric mucin secretion

in vivo systems	Agent(s)	Effects	Author
Cat stomach equipped with Heidenhanin pouch	Intravenous administration of secretin (0.5 or 3µ/kg) carbachol (10 or 20µg/kg) histamine (0.32mg/kg) pentagastrin (32µg/kg)	Stimulatory effect of all agents on the luminal content of fucose and galactose	Vagne & Perret (1976)
Laparotomized and vagotomized rat stomach	Topical and intravenous administration of prostaglandin E_2 15-methyl prostaglandin E_2 16,16-dimethyl prostaglandin E_2 (Topical, 0.13 or 1.3mg/kg; intravenous 0.6µg or 0.6 µg/kg)	Topical application of prostaglandins gave 2-3 fold and intravenous gave 1.5-2.5 fold increase in luminal alcian blue binding	Bolton *et al.*(1978)
Rat and frog stomach	Topical application of 16,16-dimethyl prostaglandin E_2 (Rat 2-10µg/ml; frog 0.5µg/ml)	In rat dose dependent increase in mucus thickness, ~80% increase at 10µg/ml. In frog ~29% increase at 0.5µg/ml.	Kerss *et al.*(1982)

continued overleaf

Table 1.2 continued

in vivo systems	Agent(s)	Effects	Author
Rat stomach	Topical administration of 16,16-dimethyl prostaglandin E_2 (25µg/kg) and intraperitoneal administration of carbachol (100µg/kg)	Prostaglandin application gave a 74% increase in gel thickness. Carbachol injection resulted in a 50% stimulation of mucus thickness	McQueen *et al.*(1984)
Rat stomach	Topical application of misoprostol (50-100µg/kg)	Approximately 3-fold increase in mucus thickness at 1000µg/kg	Sellers *et al.*(1986)
Rat stomach	Intravenous infusion of secretin (4u/kg). Topical application of 16,16-dimethylprostaglandin E_2 (5µg/ml)	Secretin infusion gave approximately 75% increase in mucus gel thickness. Prostaglandin administration gave 16-44% increase in gel thickness	Allen *et al.*(1986)
Canine stomach	Intraarterial acetylcholine chloride perfusion (50µg/ml)	10-20 fold increase in in [^3H] or [$^{35}SO_4{}^{2-}$]-labelled glycoprotein in luminal washout	Zalewsky *et al.*(1983)

continued overleaf

Table 1.2 continued

in vitro systems	Agent(s)	Effects	Author
Suspension of scraped rat gastric mucosa	16,16-dimethyl prostaglandin E_2 (10ng/ml)	32% increase in [3H]-labelled glycoprotein secretion	Slomiamy *et al.*(1987)
Suspension and culture of scraped adult rabbit mucosal cells	Biosynthetic human epidermal growth factor (1-100ng/ml)	3- fold increase in [3H]-labelled-precipitated glycoproteins at 10ng/ml EGF	Yoshida *et al.*(1987)
Culture of neonatal rat gastric mucosal cells	Arachidonic acid (10^{-4}M) glycoprotein	5- fold increase in [3H]-labelled secreted	Terano *et al.*(1987)

cells are orientated as monolayers (at confluence). These systems often require collagen or fibronectin - coated surfaces to facilitate cell attachment / polarization and in such conditions cell cultures probably secrete proteoglycans. Thus radiolabelling protocols for detecting secreted mucin may also label secreted proteoglycans. Furthermore cell cultures often use neonatal or embryonic tissue which may not possess the differentiated properties of adult tissue.

Cells scraped from the surface of the gastric muçosa (Slomiany et al., 1987; Yoshida et al., 1987) are unlike explants, slices or cultures in that intercellular contacts are largely broken. The major advantage in such preparations is the easy access of secretogogues, nutrients and oxygen to the cells. Nevertheless, mechanical rather than enzymatic tissue disruption is likely to produce a high degree of cellular damage and the probability of cellular aggregates remains high.

An isolated rat stomach cell preparation was used in this investigation to study the regulation of mucus secretion. The advantages of this system are presented in Table 1.3. Isolated cell preparations are however not without disadvantages. These include the need to use calcium chelators and proteases in cell isolation, the loss of intercellular connections and of cell polarity all of which may modify cell behaviour.

1.4.2.2 Physiological regulators of mucin secretion

Despite the evident importance of gastric mucus in protecting the epithelial surface from injury by acid and pepsin, little is known of the physiological regulation or intracellular control of mucus secretion in the stomach. Potential mucin secretogogues that have been investigated are shown in Table 1.4.

Table 1.3

Advantages of isolated cell preparations

Removal of systemic factors such as hormones, blood supply and nervous activity enables the study of single agents in isolation and the identification of their sites of action.

Nutrients and oxygen can be easily supplied.

The incubation medium can be modified enabling precise manipulation of the environment surrounding the cells.

A large number of treatments can be performed simultaneously on the same preparation.

Table 1.4

Some previously investigated potential mucin secretogogues

Agent investigated	Stimulation demonstrated as increase in Soluble mucin/sugars	Gel thickness	Radiolabelled glycoprotein *in vitro*
Acetylcholine (or muscarinic cholinergic agents)	[1] Vagne & Perret, (1976) [2] Kowalawski *et al.*, (1979)	[5] McQueen *et al.*, (1984)	–
Prostaglandin E_2 (or 16,16-dimethyl prostaglandin E_2)	[5] Bolton *et al.*, (1978) [4] Johansson & Kollberg, (1979) [5] Tao & Wilson, (1984) [5] Bersimbae *et al.*, (1985)	[4,5,6] Bickel & Kauffman, (1981) [3,5] Kerss *et al.*, (1982) [5] McQueen *et al.*, (1983) [5] Allen *et al.*, (1986)	[5] Slomiany *et al.*, (1987)
Secretin	[4] Andre *et al.*,(1972) [1] Vagne & Fargier, (1973) [1] Vagne, (1974) [1] Vagne & Perret, (1976) [2] Kowalewski *et al.*, (1976)	[5] Allen *et al.*, (1986)	

Continued overleaf

Table 1.4 continued

Agent investigated	Stimulation demonstrated as increase in: Soluble mucin/sugars	Gel thickness	Radiolabelled glycoprotein *in vitro*
Gastrin (or pentagastrin)	[1] Vagne & Fargier, (1973) [1] Vagne & Perret, (1976) [2] Kowalewski *et al.*, (1976)	–	–
Histamine	[1] Vagne & Perret, (1976) [2] Kowalewski *et al.*, (1976)	–	–
Epidermal growth factor	–	–	[7] Yoshida *et al.*, (1987)
Arachidonic acid	–	–	[5] Terano *et al.*, (1987)

Superscrips denote source of tissue as: [1] cat; [2] dog; [3] frog; [4] human; [5] rat; [6] guinea pig; and [7] rabbit.

There is a large body of evidence suggesting secretory effects in the presence of acetylcholine (and muscarinic cholinergic agonists e.g. carbachol), secretin and prostaglandin E_2. It should be noted that the majority of these reports have utilized indirect in vivo estimates of mucin secretion such as the quantitation of soluble luminal sugars/mucin or the measurement of average mucous gel thickness which imply, but do not prove, that these agents may be mucin secretogogues.

Investigation of the regulation of mucin secretion by simpler in vitro systems is largely untested and characterization of the agents which promote gastric mucin secretion from isolated mucous epithelial cells does not appear to have been previously performed.

1.5 AIMS OF THIS INVESTIGATION

This work describes the development and properties of an in vitro rat isolated gastric cell system for studying the endocrine, neurocrine and paracrine effectors which stimulate gastric mucin secretion. In particular the main aims of this work are:-

1) To develop and use a radiolabelling methodology to investigate regulation of gastric mucin secretion by isolated cells.

2) To develop and use a solid-phase enzyme immunoassay protocol to investigate the regulation of bulk gastric mucin release from isolated cells.

3) To compare the results obtained with the two procedures.

Chapter Two

GENERAL METHODOLOGY

A major portion of this thesis is concerned with the development of new methodologies to assess mucin secretion in vitro. This material is treated as results and therefore only general established methodologies are considered in this chapter.

2.1 PREPARATION OF ISOLATED CELLS

2.1.1 Preparation of an everted stomach sac

A fed male Wistar rat (200-250g body weight) was anaesthetised by an intraperitoneal injection of sodium pentobarbitone (Sagatal, May & Baker) at 60mg/kg body weight. A midline incision was made to expose the stomach. The oesophagus was ligated and the stomach was removed by cutting distal to the ligature and across the duodenum. The animal was immediately killed by puncturing the diaphragm.

The stomach was gently rinsed in ice-cold physiological saline (NaCl;9g/l) whilst holding the non-glandular region with a pair of forceps. Everted stomach sacs were then prepared (Fig 2.1) by the method of Dikstein & Sulman (1965). Medium A (Table 2.1) containing pronase at a concentration of 1000 PUK units/ml was injected into the sac using a hypodermic needle (26-gauge) until the sac was inflated; 1.5ml usually being sufficient.

2.1.2 Routine preparation of stomach fundic cell suspension

The procedure used to isolate rat fundic cells is similar to that of Trotman & Greenwell, (1979) which in turn is a modification of the method of Lewin et al., (1974). It was usually necessary to utilize four everted stomach sacs to provide sufficient cells for

Figure 2.1

Preparation of an everted fundic sac from the rat stomach.

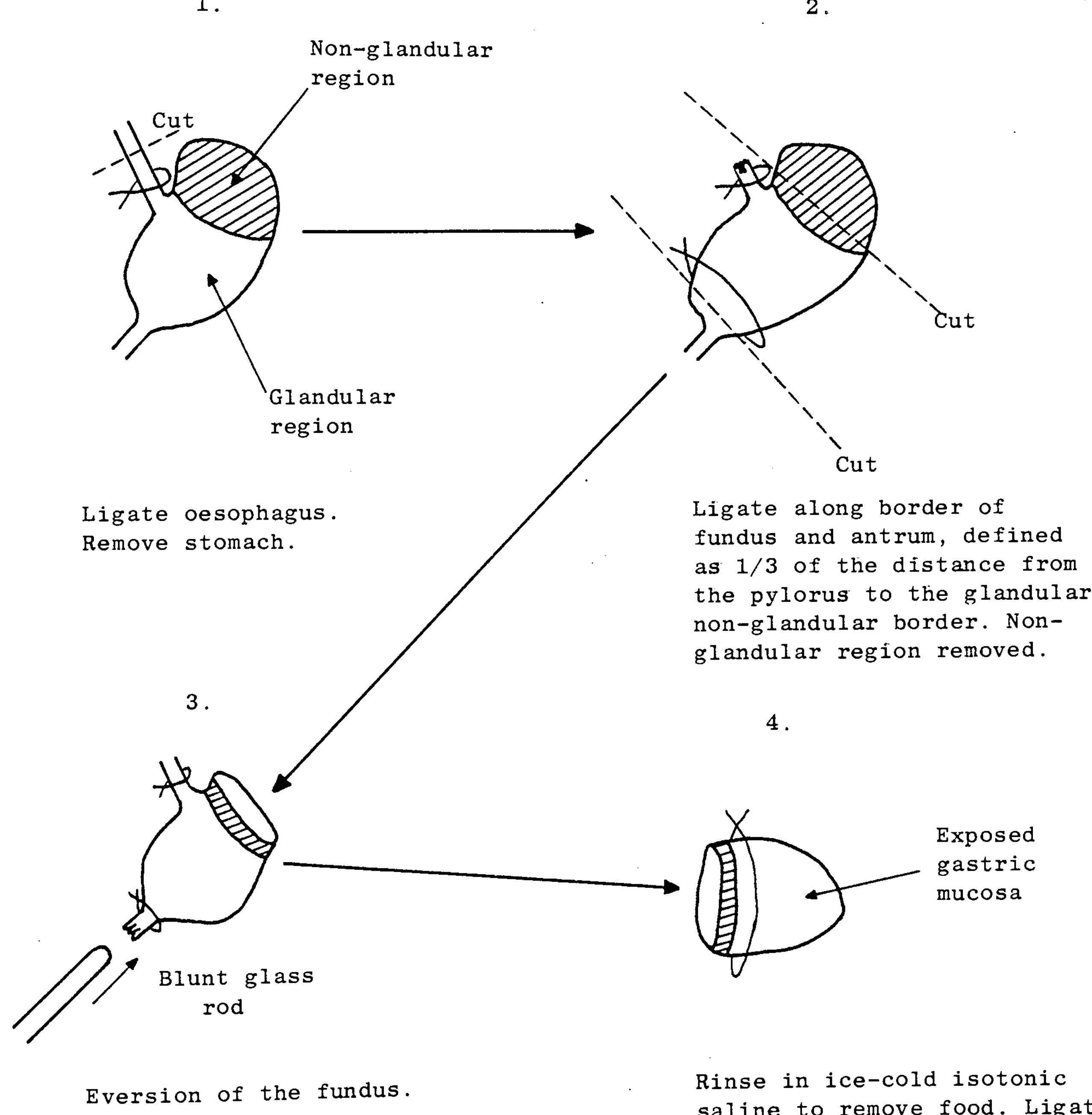


Table 2.1

Composition of media for the isolation and incubation of fundic cells.

The following additions were made to Eagle's Minimum Essential Medium (see A2) containing 25mM-$NaHCO_3$ and 20mM-HEPES.

Medium	Additions
A	EDTA (2mM) Soybean trypsin inhibitor (0.1mg/ml) Dextran (30mg/ml)
B	Bovine serum albumin fraction V (30mg/ml)
B'	Bovine serum albumin fraction V (1mg/ml)

one experiment. The sacs were placed in a plastic jar containing 40ml medium A (Table 2.1) and incubated for 30 min in a shaking water bath (140 cycles/min) at 37^0C with continuous gassing using 95% O_2/5% CO_2. This gas mixture was used throughout the experiment to gas the space over the cells. The sacs were blotted on filter paper (Whatman No 1, Whatman, Maidstone) and transferred to a plastic beaker (50ml capacity) containing 20ml medium B (Table 2.1) and sealed with laboratory film (Whatman, Maidstone).

Incubation of the sacs in medium B was for 30 min at room temperature with gassing of the airspace above the medium and the sacs were gently stirred using a magnetic follower at approximately 100 r.p.m. Cells released into medium B were filtered through nylon mesh (150µm pore size; Sericol Group Ltd, London) into 10ml plastic centrifuge tubes and were centrifuged at 125g for 7 min at 15^0C. The supernatant was discarded and the cell pellet was carefully resuspended in 10ml fresh medium B using a plastic transfer pipette (L.I.P. Ltd, Shipley,), before storage at 37^0C with shaking (140 cycles/min) and continuous gassing. The sacs were incubated for a further 2 hours with changes from incubation in medium A to cell harvesting in medium B every 30 min. The cell fractions were pooled and centrifuged at 15^0C for 7 min at 125g and the cell pellet was resuspended in 20ml of medium B'(Table 2.1). A portion (20µl) of cell suspension was removed for the assessment of cell viability (2.1.3.) prior to a second centrifugation step and resuspension in the appropriate incubation medium at a cell concentration of 10^7 cells/ml.

Preparation of isolated cell suspensions using this method typically produced a cell-fraction containing approximately 30% mucous cells (by Sigma

periodic acid/Schiff staining kit; Plate 2.1) and the average number of cells isolated per stomach was $7.68 \pm 0.18 \times 10^7$ (n=10).

2.1.3 Assessment of cell viability using the trypan blue exclusion test

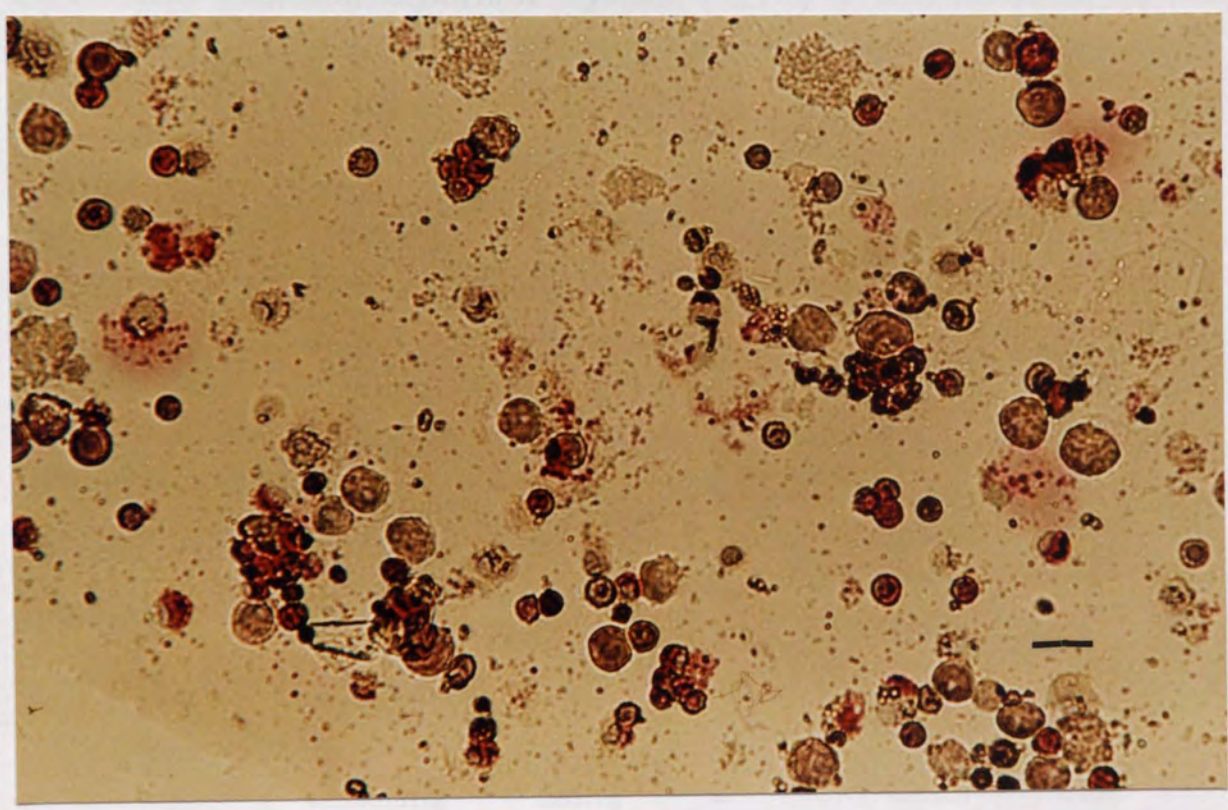
Parietal cells present in the isolated cell preparation can be identified using the light microscope based on their size. Parietal cells are the largest cell-type present, with a diameter exceeding 13µm. In this work cell viability was usually assessed for the non-parietal cell-fraction only, which probably contained predominantly mucous and chief cells.

The structural integrity of the isolated non-parietal cells was measured by their ability to exclude the dye trypan blue. An aliquot (20µl) of cell suspension was mixed well with an equal volume of trypan blue (4mg/ml) dissolved in physiological saline. An overestimate of cellular integrity may be obtained in the presence of a high concentration of bovine serum albumin which can bind trypan blue (Seglen, 1976) or if the contact time of the cells with the dye is inadequate (Elliot, 1979). In this work cell viability was assessed in medium B' which contains only 1mg/ml bovine serum albumin Cells were counted using a haemocytometer (E. Leitz, Wetzlar) by light microscopy (x400 magnification). At least 200 cells were counted on each occasion and the percentage of cells retaining the ability to exclude trypan blue was calculated. If less than 85% of cells were able to exclude trypan blue, the cell preparation was not used. Using the isolation procedure detailed in 2.1.2, 94.8 ± 0.66% (n=10 cell batches) of the whole cell preparation and

Plate 2.1

The appearance of the isolated gastric cell preparation stained using the periodic acid/Schiff stain.

Bar represents 20μm



96.9±0.54% (n=10 cell batches) of non-parietal cells were judged to be viable as determined by their ability to exclude trypan blue.

2.2 LIQUID SCINTILLATION COUNTING

2.2.1 The 'Protosol/Econofluor' system

Measurement of radioactivity associated with material precipitated by trichloroacetic acid/phosphotungstic acid from isolated stomach cells was assessed using Protosol (a tissue solubilizer) in conjunction with Econofluor (a scintillation fluid). Precipitated material was pelleted in microfuge tubes (L.I.P. Ltd, Shipley) by centrifugation at 12000g for 30 s. The tip of each tube was cut off using a heated scalpel and placed in individual glass scintillation vials. Protosol (0.5ml) was added to each vial which was incubated at 37^0C overnight. The following morning 10ml of Econofluor was added to each vial and the contents were mixed by inversion. The radioactivity of each sample was measured over 10 min by liquid scintillation counting using a TR1-CARB 2600 scintillation counter (Packard).

2.2.2 The 'Optiphase Safe' system

Measurement of radioactivity present in Superose 6 FPLC column fractions was assessed using Optiphase Safe. A portion (400µl) of each column fraction was placed in individual polyethylene scintillation vials (Pharmacia/LKB, Milton Keynes) to which 10ml Optiphase Safe was added. The contents were thoroughly mixed by inversion and the radioactivity of each sample was measured as described previously.

2.3 PREPARATION OF PARTIALLY PURIFIED RAT GASTRIC MUCIN

2.3.1 Preparation of an homogenate of scrapings from the rat gastric mucosa

A fed male Wistar rat (200-250g body weight) was anaesthetised as described earlier (2.1.1). The stomach was removed through a midline incision by cutting both the oesophagus and across the duodenum. The stomach was placed on ice, opened along the greater curvature and washed twice in physiological saline (NaCl; 9g/l).

The stomach was then placed on an ice-cold glass plate so that the exposed mucosa was uppermost. The adherent mucus was removed (together with some cells) by gently scraping the epithelial cell surface with a scalpel blade (no 10, Swann-Morton, Sheffield). The scrapings were added to extraction buffer: 0.15M-sodium chloride, 0.05M-sodium phosphate (pH7.0) 5mM-EDTA, 1μg/ml pepstatin, 1μg/ml leupeptin and 100μM-phenylmethylsulphonyl fluoride and homogenized using an Ultra-Turrax (Janke & Kunkel, Belmont) running at maximum speed for 1 min.

2.3.2 Caesium chloride density gradient centrifugation

The homogenate of gastric scrapings was centrifuged at 150C for 10 min at 1950g. Caesium chloride was added to the supernatant to give a density of approximately 1.40g/ml i.e. 40% (w/w). The caesium chloride/homogenate mixture was aportioned to polycarbonate centrifuge tubes (10ml per tube, usually 6 tubes per run) and centrifuged at 110C for 69h at 150000g on a Beckman L8-60M ultracentrige using a 70 Ti rotor.

After centrifugation the gradient was unloaded by inserting a needle down to the bottom of the tube and removing fluid by a Minipuls peristaltic pump (Gilson, Anachem) and collecting 0.5ml fractions using a Microcol TDC80 fraction collector (Gilson, Anachem).

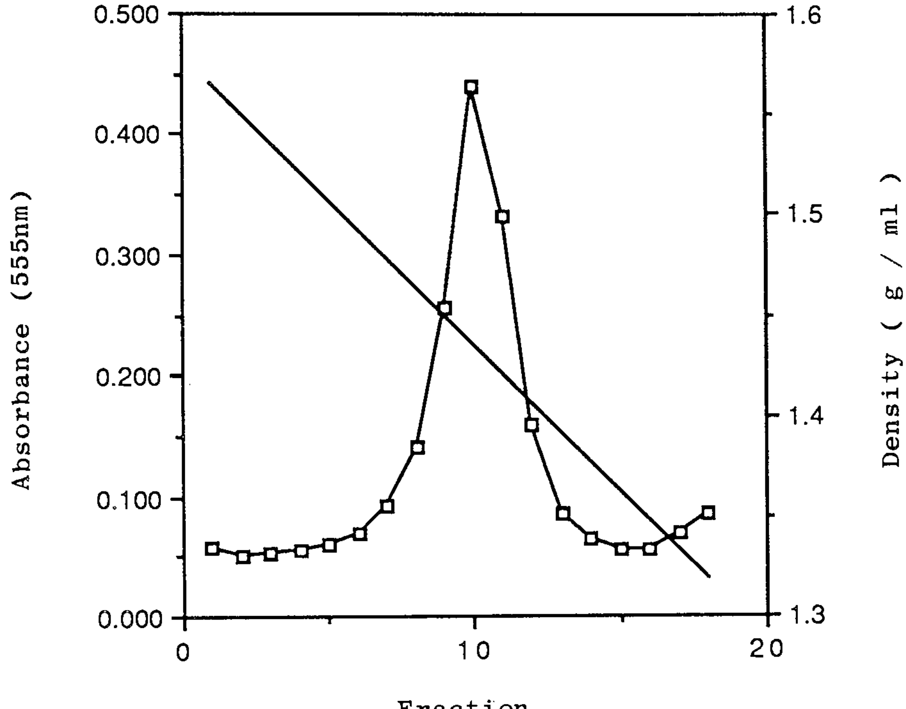
2.3.2.1 Determination of densities of fractions, absorbance (280nm) and content of rat gastric mucin

The density of each fraction was determined by weighing 200µl of solution on an electronic balance (Sartorius Handy, Sartorius). After the reading was taken the solution was returned to the tube. Absorbance at 280nm was determined using quartz cuvettes and a dual beam spectrophotometer (Pye Unican SP30, Cambridge). Measurements were made against a water blank. Fractions containing mucin were identified by using a colourimetric periodic acid/Schiff assay to measure carbohydrate (2.4). The density and glycoprotein profiles from a typical caesium chloride density gradient are presented in Fig 2.2.

Fractions containing mucin were dialysed against 3 changes of distilled water (2.5l) for 24h at 40C. An absorbance profile (200-300nm) of dialysed mucin is presented in Fig 2.3. Solution containing mucin was aportioned to LP3 (L.I.P. Ltd, Shipley) tubes and freeze-dried using an Edwards Modulyo freeze-dryer (Edwards, Crawley) overnight. Lyophilized mucin was stored at -200C.

Figure 2.2

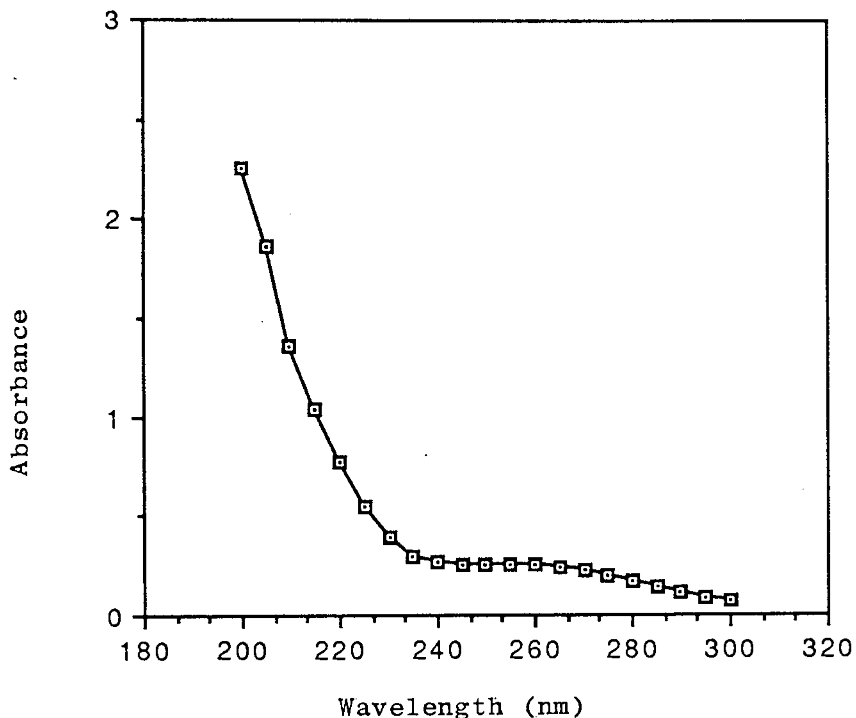
Glycoprotein and density profiles obtained from a typical caesium chloride density gradient.



Results are presented from a single experiment. The continuous straight line represents density.

Figure 2.3

Absorbance profile of dialysed gastric mucin over 200-300nm.



Results are presented from a single experiment.

2.3.3 Fast Protein Liquid Chromatography using a Superose 6 column

Further purification of mucin by FPLC gel filtration was as described in 3.2.3. Fractions 10-15 were dialysed against distilled water as described in 2.3.2.1. The mucin concentration was estimated using the periodic acid/Schiff colourimetric assay (2.4) and the mucin was freeze-dried and stored as described in 2.3.2.1.

2.4 PERIODIC ACID/SCHIFF COLOURIMETRIC ASSAY

Rat gastric mucin was detected using a modification of the method of Mantle & Allen (1978). Schiff reagent was prepared by dissolving 1g of basic Fuchsin (BDH, Poole) in 100ml of boiling water. 1M-HCl (20ml) was added to this solution which was allowed to cool to 50^{0}C. The solution was mixed with 300mg activated charcoal and shaken for 5 min. The charcoal was removed by filtering the solution through filter paper (No:1, Whatman, Maidstone) and the charcoal addition/filtration step was repeated. The resulting solution was stored in an amber glass bottle at room temperature. Immediately before use 0.1g sodium metabisulphite was added to every 6ml Schiff reagent required and this solution was incubated at 37^{0}C until it was pale yellow in colour (usually 2-3 h).

200µl of each glycoprotein-containing sample solution and 0, 25, 50, 100 and 200µg of pig gastric mucin (BDH Poole) standard dissolved in distilled water were placed in test tubes and 1.8ml of water was added. 200µl of freshly prepared periodic acid solution (10µl of 50% periodic acid solution in 10ml 7% (v/v) acetic acid) was added to each tube and the solutions were incubated at 37^{0}C for 2h. After periodate oxidation,

200µl of decolourized Schiff reagent was added to each tube and colour development was allowed to occur over 45 min. Absorbance at 555nm was measured for each sample and standard solution against water using a dual beam spectrophotometer (Pye Unican SP30, Cambridge). A correction was made for the absorbance of the reagent blank. The standard curve was as shown in Fig 2.4. The purpose of the curve was to check that the assay was functioning correctly, and to estimate the amount of mucin. The absorbance given by rat gastric mucin purified by density gradient centrifugation and FPLC was weight for weight equivalent to that given by pig gastric mucin and the latter was therefore routinely used as standard in this assay.

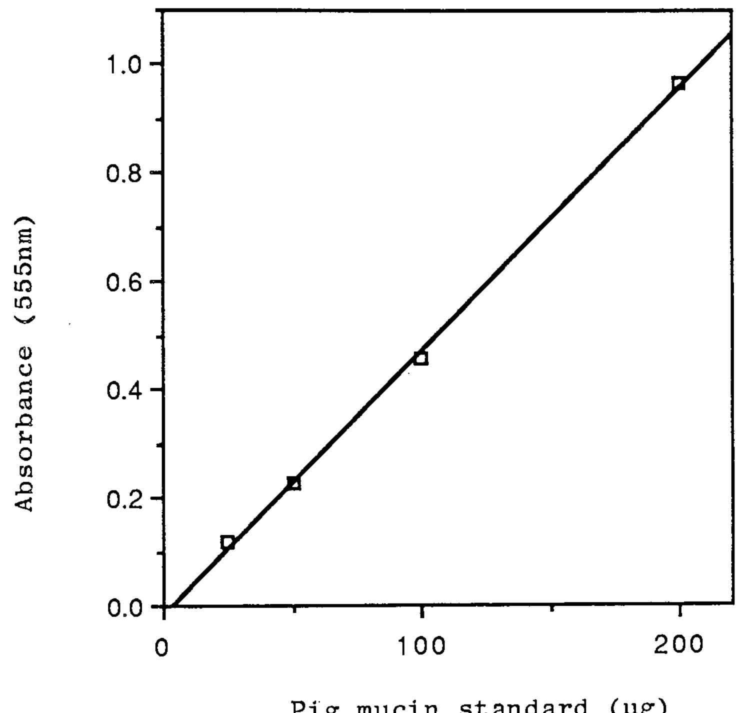
2.5 SODIUM DODECYL SULPHATE-POLYACRYLAMIDE GEL ELECTROPHORESIS (SDS-PAGE) AND SILVER STAINING

2.5.1 SDS-PAGE

Gel electrophoresis in the presence of SDS but without reduction of disulphide bonds was performed according to the method of Laemmli, (1970) using a vertical electrophoresis unit connected to a thermostatic circulator set at 10^0C (LKB, Milton Keynes). In early experiments samples were run on gels containing a 3% acrylamide stacking gel and a 7.5% acrylamide separating gel, while in later experiments a 4% acrylamide stacking gel and an 8% acrylamide separating gel was used. In all experiments, coloured protein molecular weight markers (Amersham International, Amersham) were run (Fig 2.5).

Figure 2.4

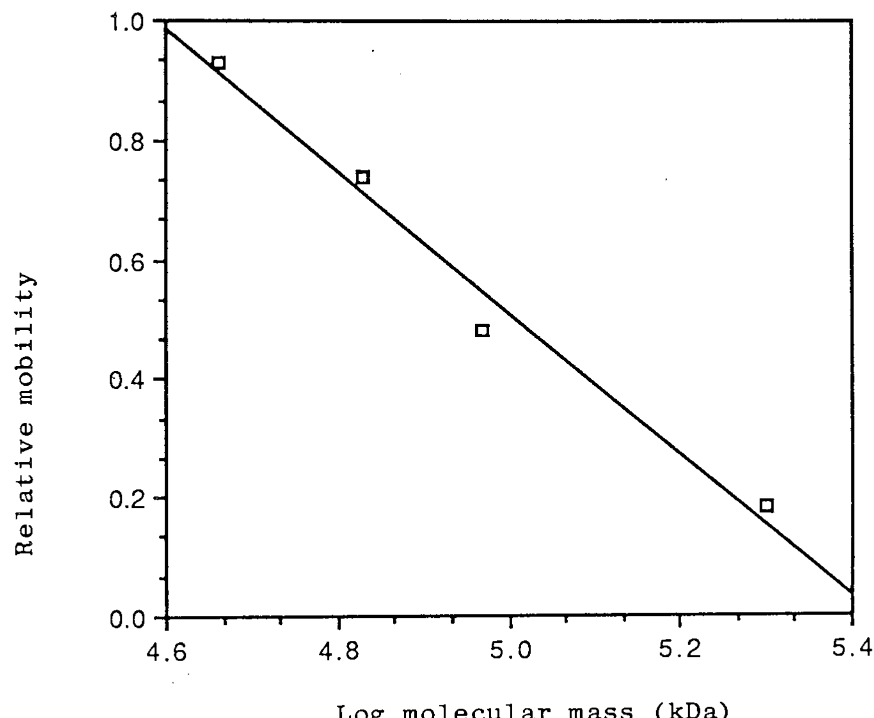
Standard curve obtained using periodic acid/Schiff colourimetric glycoprotein assay.



Results are presented from a single experiment. The equation of the regression line is y=-0.016+4.867x and the correlation coefficient, r=1.0.

Figure 2.5

Relationship between the relative mobility of coloured molecular weight markers and molecular mass.



Results are presented from a single experiment in which coloured marker proteins were run on a 7.5% acrylamide separating gel and a 3% acrylamide stacking gel. The equation of the regression line is y=6.452-1.188x and the correlation coefficient, r=0.99. The markers (kDa) were: myosin (207), phosphorylase b (97), bovine serum albumin (66) and ovalbumin (45).

2.5.2 Silver staining

Silver staining of gels was performed according to the method of Morrisey, (1981). Briefly, after electrophoresis the gel was fixed in 50% (v/v) methanol, 10% (v/v) acetic acid for 30 min, followed by washing in 5% (v/v) methanol, 7% (v/v) acetic acid for a further 30 min. After washing the gel was incubated in 10% (v/v) glutaraldehyde for 30 min, after which it was washed in three changes of distilled water overnight with agitation. Next morning the gel was soaked in 5µg/ml dithiothreitol for 30 min. This solution was decanted and without rinsing the gel was incubated with 0.1% (w/v) silver nitrate for 30 min. After incubation the gel was rinsed with a small amount of distilled water, then twice with a small amount of developer: 50µl of 37% (v/v) formaldehyde in 100ml 3% (w/v) sodium carbonate. The gel was then soaked in developer until the desired level of staining was obtained. The reaction was stopped by the addition of 5ml of 2.3M-citric acid. The developer/citric acid solution was then discarded and the gel washed with distilled water.

2.6 ASSAY OF PROTEIN

Determination of protein concentration was by the method of Bradford, (1976).

2.6.1 Preparation of reagent and of protein standards

Concentrated dye reagent (Bio-Rad, Watford) was diluted five-fold immediately before use with distilled water and filtered (No. 1, Whatman, Maidstone) into a glass container.

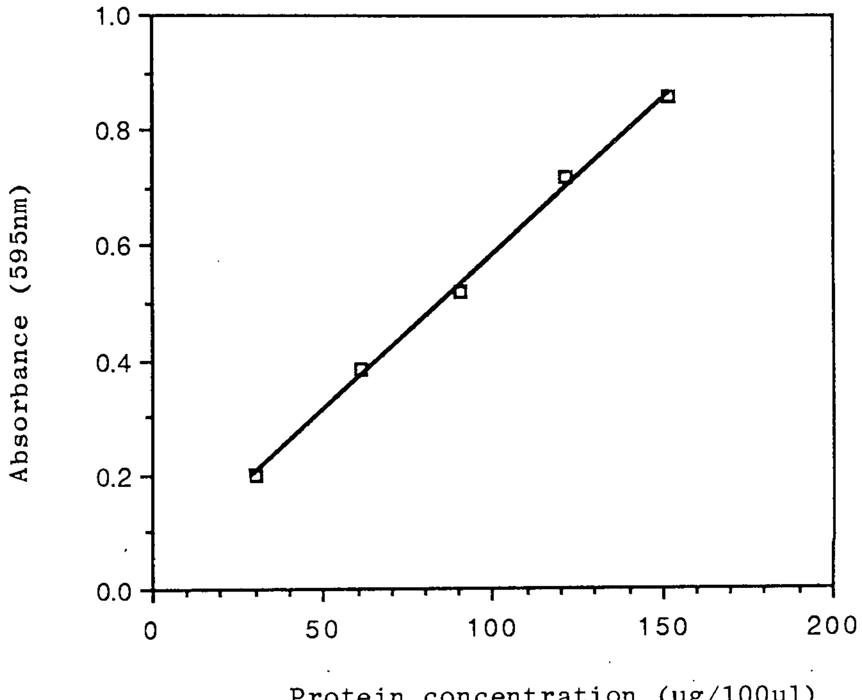
Lyophilized bovine gamma globulin standard (Bio-Rad, Watford) was reconstituted with distilled water to yield a concentration of 1.52mg/ml, at stored at 4^0C.

2.6.2 Assay procedure

Several dilutions of the protein standard (0.30, 0.61, 0.91, 1.22, 1.52 mg/ml) were prepared. 100µl of the standards, samples and a buffer blank were placed in separate test tubes and 5ml of diluted dye reagent was added to each tube. The contents of each tube were mixed using a vortex mixer (avoiding excess foaming). After a period of 10 min the absorbance of each tube at 595nm was measured against water using a dual beam spectrophotometer (Pye Unican SP30, Cambridge). A correction was made for the buffer blank and the standard curve was as shown in Fig 2.6.

Figure 2.6

Standard curve obtained using Bradford protein assay.



Results are presented from a single experiment. The equation of the regression line is y=0.042+0.005x and the correlation coefficient, r=1.0.

Chapter Three

DEVELOPMENT OF A RADIOCHEMICAL ASSAY TO QUANTITATE THE SECRETION OF RAT GASTRIC MUCIN.

3.1 INTRODUCTION

3.1.1 Isotopic precursors used in the labelling of gastrointestinal mucin

Incorporation of radiolabelled precursors into mucin has been widely used in *in vitro* investigations of the biosynthesis and secretion of gastrointestinal mucin. Precursors that have been utilized in such studies are listed in Table 3.1. In this work D-[^{3}H]glucosamine was used to label rat gastric mucin. The properties and suitability of each type of compound for labelling mucin to high specific activity are outlined and compared to the selected label below. The three most important factors are proportion of mucin by weight labelled by the precursor, its tendency to label substances other than mucin and the specific activity of the precursor. The first two of these will be discussed below, whilst precursor specific activities are presented in Table 3.1.

3.1.1.1 [^{3}H] or [^{14}C]-labelled sugars

The most commonly used precursors which are incorporated into the carbohydrate moiety of mucin are D-glucosamine, D-glucose and D-galactose. Radioactive D-glucosamine is not directly incorporated into mucin but is acetylated to N-acetyl-D-glucosamine which is then incorporated into mucin via nucleotide activation (Carlstedt *et al.*, 1985). N-acetyl-D-glucosamine has been shown to represent approximately 16% and 20% of mucin glycoprotein by weight in the rat (Slomiany *et al.*, 1987) and pig (Scawen & Allen, 1977) respectively and thus provides a large target for radiolabelling. Although D-glucosamine is an intermediate in mucin biosynthesis it can be metabolized into other cellular components. Thus D-glucosamine can be phosphorylated and deaminated to yield fructose-6-phosphate an intermediate in the glycolytic pathway (Carlstedt *et al.*, 1985).

Table 3.1

Some investigations of gastrointestinal mucin using radiolabels and the specific activity at which they are currently available

Authors	Tissue studied	Radiolabels used	Maximum Specific activity (Amersham)
Tairov *et al.*, (1984)	Rat gastric mucosa	[^{3}H]N-acetylglucosamine	2-10 Ci/mmol
Slomiany *et al.*, (1985)	"	[^{3}H] proline [^{3}H] palmitate	100-130 Ci/mmol 40-60 Ci/mmol
Jentjens *et al.*, (1986)	Rat fundic gastric mucosa	[^{35}S] cysteine	>1000 Ci/mmol
Spohn & McColl, (1987)	Human antral gastric mucosa	[U-^{14}C] leucine [U-^{14}C] glucose	>300mCi/mmol >230mCi/mmol
van Beurden Lamers *et al.*, (1989)	Rat fundic gastric mucosa	[^{3}H]galactose [^{35}S]sulphate	20-40 Ci/mmol 20-40 Ci/mg
Hunter *et al.*, (1989)	Rat stomach, duodenum and colon mucosa	[^{3}H]glucosamine [^{3}H]galactose	20-40 Ci/mmol 20-40 Ci/mmol
Heim *et al.*, (1990)	Pig gastric mucosa	[^{3}H]leucine [^{14}C]N-acetylglucosamine	120-190 Ci/mmol 2-10 Ci/mmol

Perhaps the most unsuitable of the commonly used precursors is D-glucose. D-glucose itself is not directly incorporated into mucin-type glycoconjugates and is probably metabolized to galactose (Carlstedt et al., 1985). Although D-glucose can theoretically be incorporated into a large proportion of mucin by weight, due to its central role in cellular metabolism it is likely to be incorporated into all the rapidly synthesized components of the gastric mucosa (Spohn & McColl, 1987) and therefore is unlikely to be as effective a label as D-glucosamine. Clearly, protocols involving radioactive D-glucose will also require extensive sample purification before all labelled material can be taken to be mucin.

The use of D-galactose to label mucin is widespread since this sugar is incorporated via nucleotide activation directly into the carbohydrate moiety. It has been estimated to represent approximately 22% and 26% by weight of mucin in the rat (Slomiany et al., 1987) and pig (Scawen and Allen, 1977), a similar proportion to that of N-acetyl-D-glucosamine. D-galactose can also similarly to D-glucosamine be incorporated into other cellular components. Thus UDP-galactose can isomerize to UDP-glucose (Carlstedt et al., 1985). Therefore in experiments using glucosamine or galactose not all of the label can be expected to end up in glycoproteins. D-glucosamine was selected over galactose since the latter was thought to be potentially more likely to enter the glycolytic pathway than the former.

It should be emphasized that the use of a [^{3}H] label rather than a [^{14}C] label is a compromise between label detectabilities and their relative costs. Although [^{3}H] counting efficiences by liquid scintillation counting are in the order of half of [^{14}C], the relative cost of the [^{14}C] label is approximately 5-fold more per µCi (Amersham International, Amersham).

3.1.1.2 [^{3}H], [^{14}C] and [^{35}S] - labelled amino acids

Radioactive amino acids commonly incorporated into the central peptide core of gastric mucin are L-proline, L-serine and L-cysteine. The content of proline, threonine and serine in rat gastric mucin is quite high (approximately 9, 16 and 15 residues / 100 residues; Dekker et al., 1989b). In pig gastric mucin these amino acids have been shown to represent 49.2 moles/100 moles of amino acid (Scawen & Allen, 1977). The content of cysteine in rat gastric mucin is low (approximately 1 residue / 100 residues; Dekker et al., 1989b), whilst pig gastric mucin contains approximately 3.1 moles / 100 moles of amino acid (Scawen & Allen, 1977). Cysteine can be labelled with [^{35}S] and the high energy of the ß-emission can make this the best precursor if the peptide backbone is to be detected on SDS-polyacrylamide gels by autoradiography. Otherwise labelling with either proline or serine would seem preferable, although the low content of protein (typically 20% by weight) in mucin makes radioactive amino acids less satisfactory than sugars for labelling mucin. Furthermore, amino acids will be incorporated into all rapidly synthesized cellular proteins in the gastric mucosa.

3.1.1.3 [$^{35}SO_4{}^{2-}$]

Sulphate is a component of most gastrointestinal mucins (Forstner et al., 1973) being added to oligosaccharide chains from the activated sulphate donor PAPS (3'-phosphoadenosine-5'phosphosulphate). Ester-bound sulphate is a relatively minor component (approximately 3% by weight; Slomiany et al., 1987) of rat gastric mucin. Thus [$^{35}SO_4{}^{2-}$] is unlikely to produce labelled mucin of high specific activity. In addition, only acidic mucins will be labelled. Incorporation of [$^{35}SO_4{}^{2-}$] is unlikely to label all mucin equally and the results may not therefore truly reflect bulk mucin metabolism.

3.1.1.4 [^{3}H]-palmitate

Covalently bound fatty acid, particularly palmitic acid has been detected in trace amounts in rat gastric mucin where it represents approximately 0.4% by weight (Slomiany et al., 1987) of mucin. Metabolic labelling with [^{3}H]palmitate would not therefore be expected to provide adequate labelling of the intracellular mucin pool.

3.1.2 Fast protein liquid chromatography (FPLC)

3.1.2.1 Gel filtration

Gel filtration chromatography separates molecules on the basis of their molecular size and shape. The column materials possess a three-dimensional network of pores, which act as gates permitting small molecules to enter but excluding larger molecules. These gating effects represent a continuous decrease in accessibility for molecules of increasing size.

Common materials used in gel filtration include cross-linked dextrans (trade name Sephadex), agarose (Sepharose, Bio-Gel A) and polyacrylamide (Bio-Gel P). Since gastrointestinal mucins have considerable molecular mass, these molecules are easily separated from smaller proteins by gel filtration and this technique has been widely used (Table 3.2). Elution of mucins usually occurs close to or in the void volume.

3.1.2.2 The Superose 6 FPLC column

The technique of fast protein liquid chromatography (FPLC) is essentially an adaption of high performance liquid chromatography (HPLC). The equipment required is similar to that for HPLC except most components are constructed from glass and plastic rather than metal. Superose 6 (Table 3.3) is a gel filtration medium formed from cross-linked agarose which was designed specifically for chromatography

Table 3.2

Gel filtration media used in investigations of gastrointestinal mucin

Media	Material	Useful fractionation range of globular proteins (Da)	Authors
Bio-Gel P-100	Polyacrylamide	$5x10^3 - 1x10^5$	Slomiany et al., (1984)
Bio-Gel A-15m	Agarose	$4x10^4 - 1.5x10^7$	Tsurui et al., (1986) Ohara et al., (1988)
Bio-Gel A-50m	Agarose	$1x10^5 - 5x10^7$	Slomiany et al., (1984)
Sepharose 2B	Agarose	$7x10^4 - 4x10^7$	Pearson et al., (1980) Pearson et al., (1981) Hunter et al., (1989)
Sepharose 4B	Agarose	$6x10^4 - 2x10^7$	Lukie & Forstner, (1971) Seidler & Sewing, (1988) Seidler & Sewing, (1989)
Sepharose CL-2B	Cross-linked agarose	$7x10^4 - 4x10^7$	Bagshaw et al., (1987) Dekker et al., (1989)
Sepharose CL-4B	Cross-linked agarose	$6x10^4 - 2x10^7$	Spee-Brand et al., (1980)

Table 3.3

Properties of the Superose 6HR 10/30 column

Property	Superose 6
Exclusion limit (globular proteins) (Da)	~ 4×10^7
Useful separation range (globular proteins) (Da)	$5 \times 10^3 - 5 \times 10^6$
Material	Cross-linked agarose
Average particle size (μm)	13±2
Loading capacity	5-10mg protein in 200μl

under back-pressures of approximately 1 bar and is available commercially in prepacked columns (Pharmacia - LKB, Milton Keynes).

FPLC with Superose 6 has two major advantages over conventional low pressure or gravity gel filtration with, for instance Sepharose columns. Firstly, separation times with Superose 6 are typically only 40 min, whereas Sepharose requires typically 5-24 h per run. Secondly, the smaller particle size distribution of Superose 6 confers much greater resolution than is possible with conventional gel filtration.

Since several samples of incubation medium were likely to be analysed for released mucin, separation by FPLC using Superose 6 was selected because of its better resolution and speed by comparison with conventional low pressure chromatography.

3.1.3 Aims of this work

The specific aims of this section were:

1) To establish a protocol using [^{3}H]glucosamine to label mucin in a suspension of epithelial cells isolated from rat gastric mucosa.

2) To develop a gel filtration method using a Superose 6 FPLC column to rapidly separate rat gastric mucin secreted into the medium of an isolated cell preparation from other labelled material.

3) To establish that the labelled material isolated by FPLC was mucin.

3.2 METHODOLOGY

3.2.1 Measurement of D-[6-^{3}H]glucosamine uptake by isolated stomach cells

Cells were isolated from the stomachs of two rats as described previously (2.1), and were washed once with 10ml medium B'. The cells were resuspended in medium B' containing 8 µg/ml insulin, 10nM-hydrocortisone, 5% (v/v) foetal calf serum, 0.5mM-dithiotheritol, 2µCi/ml D-[6-^{3}H]glucosamine, 1mM-glutamine, 50µg/ml gentamicin at a cell concentration of 10^{7} cells/ml and incubated at 37^{0}C for 2 h. The incubation flask (50ml ; Nalgene, BDH, Poole) was gassed continuously with 95% 0_2 / 5% $C0_2$ and was shaken at 140 cycles/min.

Duplicate samples of incubation medium (0.5ml) were removed from the incubation flask at 20 min intervals and immediately centrifuged (12000g for 10 s). The supernatants were discarded and the cells resuspended in incubation medium without the D-[6-^{3}H] glucosamine. The suspensions were recentrifuged and the supernatant fractions discarded. The pellets were resuspended in 1ml ice-cold 10% (w/v) trichloroacetic acid/0.5% (w/v) phosphotungstic acid (TCA/PTA) solution using a vortex mixer, and left on ice for 30 min. Precipitates were collected by centrifugation (12000g x 2min) and the supernatants discarded. The pellets were resuspended in 1ml ice-cold TCA/PTA using a vortex mixer and recentrifuged. The precipitates were washed again and finally the radioactivity was counted using the Protosol/Econofluor system (New England Nuclear, Southampton; see 2.2.1).

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3.2.2 Protocol for pulse / chase radiolabelling of isolated stomach cells with D-[6-^{3}H]-glucosamine

Cells required for pulse/chase radiolabelling experiments were isolated from the stomachs of four rats as described previously (see 2.1). Cells were incubated at 37^{0}C in Medium B' containing 1mM-glutamine, 50µg/ml gentamicin, 5% (v/v) foetal calf serum, 8µg/ml insulin, 10nM-hydrocortisone and 10µCi/ml D-[6-^{3}H]-glucosamine for 2 h. The pH of this medium was 7.4 and the cell concentration was 10^7 cells/ml. The incubation flask (50ml) was shaken at 140 cycles/min and was continuously gassed with 95% 0_2 / 5% $C0_2$.

After the incubation period, the cell suspension was transfered to 10ml polystyrene tubes by using a plastic transfer pipette and was centrifuged at 120g for 7 min at 15^{0}C (MSE Chilspin, Crawley). The supernatants were discarded and the cell pellets were resuspended in medium B' and recentrifuged as above. The wash supernatants were discarded and the pellets were resuspended and incubated (under the same conditions) for a further hour in a medium the same as the initial incubation medium except that D-[6-^{3}H]glucosamine was replaced by unlabelled 1mM-D-glucosamine and 0.5mM-dithiothereitol was added.

The cells were then washed as before, and the pellet was resuspended with medium B' containing 1mM-glutamine, 50µg/ml gentamicin and usually 0.5mM-dithiothreitol. In experiments using the secretogogue combination 0.5mM-carbachol and 1mM-dibutyryl cyclic AMP (dbcAMP) these agents were added to the resuspension medium. The cell suspension was then aliquoted (in 2ml portions) to polyethylene scintillation vials. (Pharmacia-LKB, Milton Keynes). The air space was gassed with 95% 0_2 / 5% $C0_2$ and the

vials capped. Release of $[^3H]$-labelled material from the cells was assessed after incubation at 37^0C and shaking at 150 cycles/min.

3.2.3 Gel filtration by FPLC

3.2.3.1 Preparation of secreted $[^3H]$-labelled material

Samples of cell suspension were transferred from the scintillation vials to microfuge tubes and were centrifuged at 12000g for 30 s. The supernatant fractions were transferred to Centricon 30 microconcentrators (Amicon, Stonehouse, Gloucestershire) and were centrifuged at 2000g for 30 min at room temperature (until <100µl remained). Material of molecular mass (kDa) greater than 30 kDa, which was present in the Centricon retentate, was reconstituted to 2ml in a medium (pH 7.0) containing: 0.15M-NaCl, 0.05M-sodium phosphate, 0.02% (w/v) sodium azide and 0.5% (w/v) 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulphonate (CHAPS). This material was stored in capped microconcentrators at 4^0C, and was concentrated by recentrifugation. The material was made up to 250µl with the above medium and was centrifuged at 12000g for 10 min prior to analysis by gel filtration.

3.2.3.2 Preparation of $[^3H]$-labelled cellular material

A sample of cell suspension (2ml) obtained before the measurement period was transferred to plastic microfuge tubes (2x1ml) and was centrifuged at 12000g for 30 s. The supernatants were discarded. Cell pellets were sonicated for six 5 second periods at 20W (MSE Soniprep, Croydon) in 1ml extraction medium (pH 7.0) containing 0.15M-NaCl, 0.05M-sodium phosphate, 0.1mM-EDTA, 1µg/ml pepstatin, 1µg/ml leupeptin and 100µM-PMSF. The sonicated pellets were allowed to stand for 1 h at room temperature. Then 0.5ml of extraction medium containing 15% (w/v) CHAPS was added to give a final CHAPS concentration of 5% (w/v). The

mixture was stored at 4^0C and was centrifuged at 12000g for 10 min and filtered (0.45µm) prior to analysis by gel filtration.

3.2.3.3 Protocol for gel filtration

Fast protein liquid chromatography (Fig. 3.1) was performed using a Superose 6 column (Pharmacia-LKB, Milton Keynes). Prior to sample application, the column was equilibrated with degassed and filtered (0.45µm) elution buffer (0.15M-sodium chloride, 0.05M-sodium phosphate, 0.5% (w/v) CHAPS, pH 7.0) at a flow rate of 0.25ml/min until at least 50ml effluent had passed through. Experimental samples (200µl) were subsequently run at 0.25 ml/min with fractions collected every 2.5 min (40 in total; Microcol TDC 80, Gilson). Absorbance was monitored at 280nm (Uvicord SD, LKB, Milton Keynes). [^{3}H]-labelled material in the fractions was detected by scintillation counting using Optiphase Safe (Pharmacia-LKB, Milton Keynes; see 2.2.2).

3.2.4 Binding assay for intrinsic factor

Intrinsic factor was detected as [^{57}Co]cyanocobalamin binding activity (Schepp *et al.*, 1983).

3.2.4.1 Protocol for binding assay

Material secreted into the supernatant fraction of the rat stomach cell suspension was prepared for gel filtration and chromatographed as described in 3.2.3.1 & 3.2.3.3., except CHAPS was omitted from these procedures. Preparation of cellular material was similar to that described in 3.2.3.2 except that cells were sonicated into medium B'.

A portion (10µl) of each fraction of chromatographed supernatant material was added to 90µl phosphate buffered saline (PBS; 0.15M-sodium chloride,

Figure 3.1

Schematic representation of the isocratic FPLC system used in this work.

Solvent reservoir

LKB 2150 HPLC pump

LKB 2152 HPLC controller

Injection loop

Superose 6 column

LKB 2158 HPLC detector

Phillips PM 8251 chart recorder

Gilson TDC 80 fraction collector

0.05M-sodium phosphate, pH 7.0) in a microfuge tube. To this 100µl of [^{57}Co]cyanocobalamin label containing 10^5 cpm was added and the mixture was incubated for 15 min at room temperature. Addition of 1ml albumin-coated charcoal (5% (w/v) Norit 'A' charcoal, 1% (w/v) bovine serum albumin in distilled water) was followed by a further incubation for 15 min at room temperature. The microfuge tubes were then centrifuged at 12000g for 2 min and 750µl of the supernatant was assayed for [^{57}Co] by gamma scintillation counting using an LKB 1282 compugamma counter (LKB, Milton Keynes).

Total cellular binding activity was assessed in 7.5µl of cellular extract. This material was diluted to 100µl with medium B' and treated as described above.

3.2.5 Incubation of purified material with enzymes

3.2.5.1 Preparation of purified [^{3}H]-labelled high molecular mass material

Labelled material secreted by rat gastric cells incubated with 0.5mM-carbachol and 1mM-dibutyryl cyclic adenosine monophosphate (dbcAMP) was obtained as fractions 10-15 by the FPLC procedure (3.2.3.1). The fractions were pooled and concentrated using microconcentrations (until <100µl remained). The retentate was reconstituted to 600µl with PBS containing 0.02% (w/v) azide and was capped and stored at 4^0C.

3.2.5.2 Protocol for incubation with papain (E.C. 3.4.22.2)

Digestion of partially purified unlabelled rat gastric mucin (see 2.3 for preparation) and [^{3}H]-labelled high molecular mass material with papain was performed as described by Pearson et al., (1980). Papain (50µg/ml) was dissolved in 0.1M-citrate, 1mM-EDTA, 5mM-cysteine, pH 5.6 and activated by preincubation in a sealed polystyrene tube (LP3;

Luckham) for 30 min at 60^0C on a heating block. Purified, concentrated, [^{3}H]-labelled high molecular mass material (100µl), or 800µg of unlabelled partially purified mucin were added to this solution and were incubated for 48 h at 60^0C. The solutions were then centrifuged at 2000g for 25 min at 15^0C in a microconcentrator, and the retentates were analysed by FPLC on Superose 6. [^{3}H]-labelled material in the eluate was detected by scintillation counting, and unlabelled glycoprotein was detected by using the periodic acid/Schiff colourimetric assay (2.4).

The procedure used for incubation with hyaluronidase and chondroitinase ABC was similar except for the details mentioned in the following sections.

3.2.5.3 Protocol for incubation with hyaluronidase (E.C. 4.2.2.1)

Digestion of [^{3}H]-labelled high molecular mass material with hyaluronidase (Sigma Type 1X) from Streptomyces hyalurolyticus was as described by Slomiamy et al., (1987). Concentrated [^{3}H]-labelled material (100µl) was added to 1ml hyaluronidase digestion medium:- PBS adjusted to pH 6.0 with 0.1M-citric acid containing 10U/ml hyaluronidase. This solution was incubated in a sealed LP3 tube for 16 h at 37^0C on a heating block.

3.2.5.4 Protocol for incubation with chondroitinase ABC (EC 4.2.2.4)

Incubation of [^{3}H]-labelled high molecular mass material with chondroitinase ABC from Proteus vulgaris was performed as described by Slomiany et al., (1987). A portion (100µl) of concentrated [^{3}H]-labelled high molecular mass material was added to 1ml chondroitinase digestion buffer:- 0.1M-Tris-acetate, pH 7.3 containing 0.4U/ml chondroitinase ABC. This solution was incubated for 16 h at 37^0C on a heating block in a sealed LP3 tube.

3.2.6 Protocol for incubation with 0.1M-dithiothreitol

A portion (100µl) of concentrated purified [^{3}H]-labelled high molecular mass material was added to 200µl of PBS containing 0.15M-dithiothreitol. (Thus, on addition final concentration was 0.1M). This solution was incubated for 24 h at 37^{0}C in a sealed microfuge tube using a water-bath. The microfuge tube was then centrifuged at 12000g for 10 min and 200µl of the supernatant was chromatographed as described in 3.2.3.3.

100µg of partially purified, unlabelled mucin was reconstituted to 225µl in PBS. To this 25µl of PBS containing 1M-dithiothreitol was added, thus the final dithiothreitol concentration was 0.1M. This solution was incubated and subsequently run on the Superose 6 column as described above.

3.2.7 Caesium chloride density gradient centrifugation

3.2.7.1 Sample preparation

FPLC fractions 10-15 containing [^{3}H]-labelled high molecular mass material prepared from two incubation vials was finally reconstituted to 1ml in extraction medium (3.2.3.2) by using Centricon 30 microconcentrations. This solution was added to 9ml extraction medium containing caesium chloride to give a starting density of 1.45g/ml. High molecular mass, [^{3}H]-labelled, cellular material was prepared similarly to secreted material.

Freeze-dried unlabelled, rat gastric mucin (200µg by periodic acid/Schiff assay using pig gastric mucin (BDH) as standard; see 2.4 for preparation) was reconstituted to 1ml in extraction medium and added directly to 9ml extraction buffer / caesium chloride solution as described earlier.

3.2.7.2 Formation, unloading and analysis of the caesium chloride density gradient

Samples were centrifuged for 69 h at 150,000g at 10^{0}C using a 70 Ti rotor in a Beckman L8-60M ultracentrifuge. The samples were unloaded using a peristaltic pump (Gilson Minipuls, Anachem) connected to a fraction collector (Gilson Microcol TDC 80, Anachem), to give 0.5ml fractions. The density of each fraction was assessed by measuring the weight of a 200µl sample. [^{3}H]-labelled material and carbohydrate-containing material were detected in fractions by using the same procedures as those used to assay fractions from the FPLC column.

3.3 RESULTS AND DISCUSSION

3.3.1 D-[6-^{3}H]glucosamine labelling of isolated stomach cells

Incorporation of [^{3}H]glucosamine into TCA/PTA-precipitable material was linearly related to incubation time over 20 - 120 min (Fig. 3.2). Regression analysis of the data gave a correlation coefficient of 0.996, an intercept value of -817.67 and a regression coefficient of 64.57 ($p<0.001$, t-test). Linear incorporation of [^{3}H]glucosamine suggests that there was no gross change in cell viability, since loss of viability would be expected to have reduced the rate of incorporation of label. Indeed, essentially linear incorporation of radioactive tracers over at least 16 hours has been reported by Seidler & Sewing, (1989) using rabbit fundic explants and Heim et al., (1990) using pig isolated gastric mucosal cells.

In conclusion cell suspensions incubated with D-[6-^{3}H]glucosamine for 2h appeared to incorporate label into material precipitated by TCA/PTA. The nature of this material is discussed later (3.3.3).

3.3.2 Gel filtration profile of secreted and cellular [^{3}H]-labelled material

3.3.2.1 Description of peaks

Isolated cell suspensions were incubated with 0.5mM-carbachol plus 1mM dbcAMP in an attempt to obtain maximal release of mucin should the cells prove responsive to stimulation (Fig 3.3A, open symbols). Two major components of [^{3}H]-labelled material were identified by Superose 6 analysis of medium obtained from such suspensions. A sharp peak of high molecular mass, [^{3}H]-labelled, material (henceforth denoted as Peak-1) eluted from the column close to the void volume and before thyroglobulin (669 KDa; see Fig 3.4 for elution of marker compounds from the column). The

Figure 3.2

Relationship between incubation time and incorporation of radiolabel into TCA/PTA precipitable material from an isolated cell suspension.

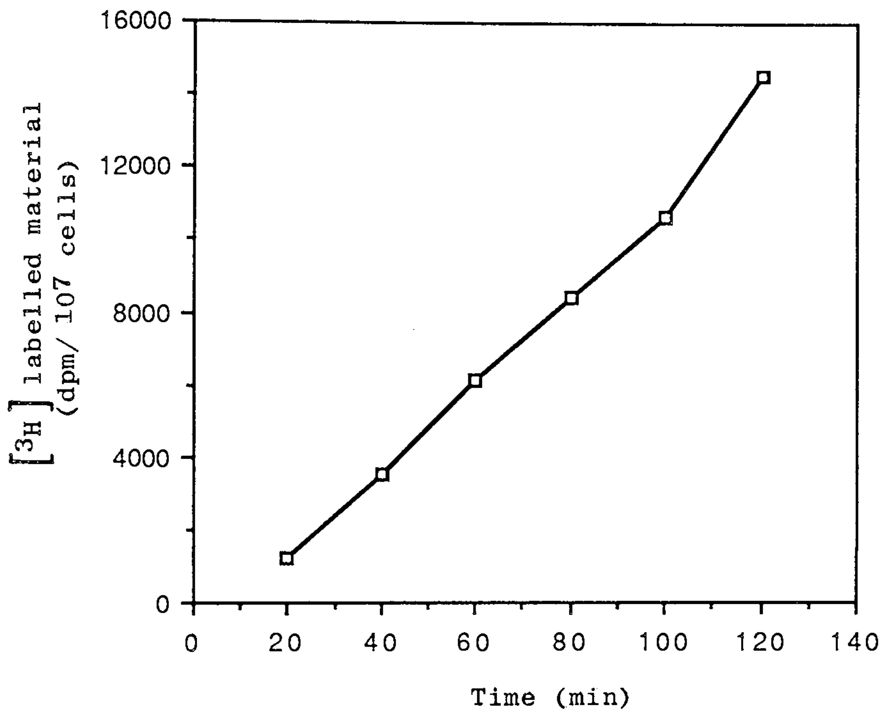
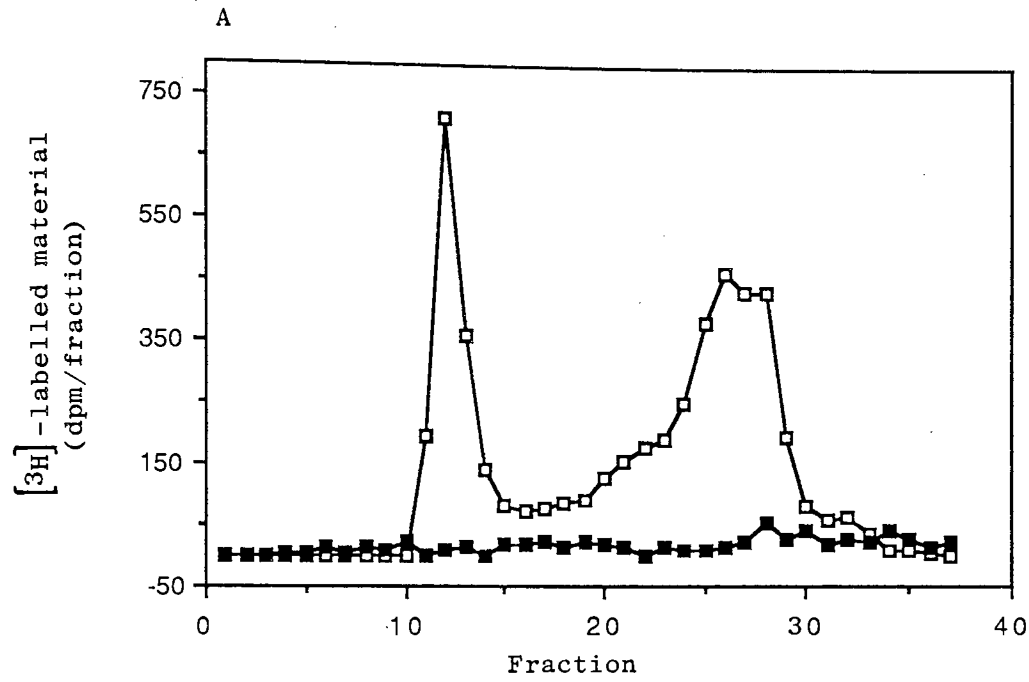
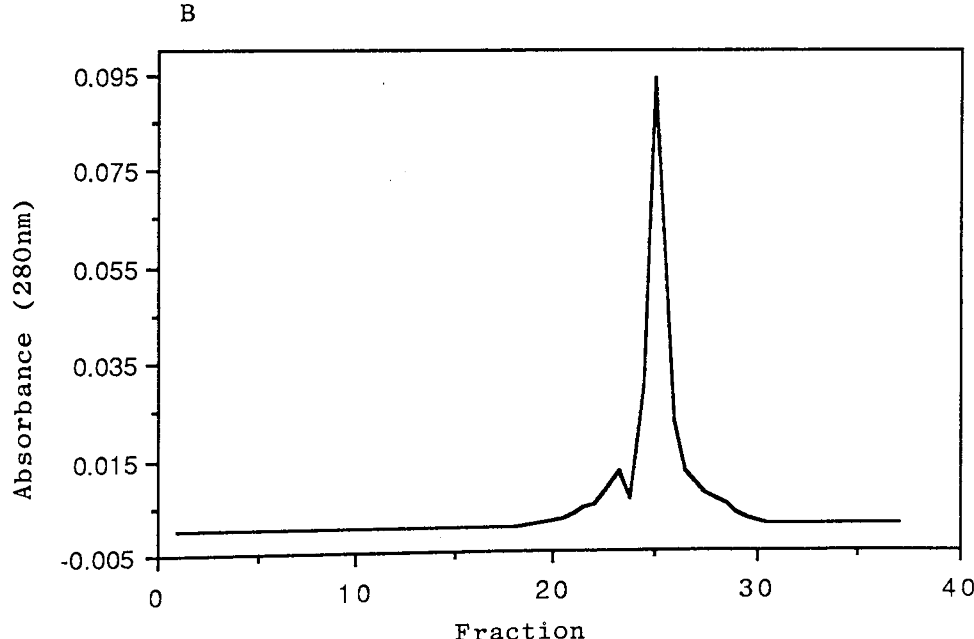


Figure 3.3



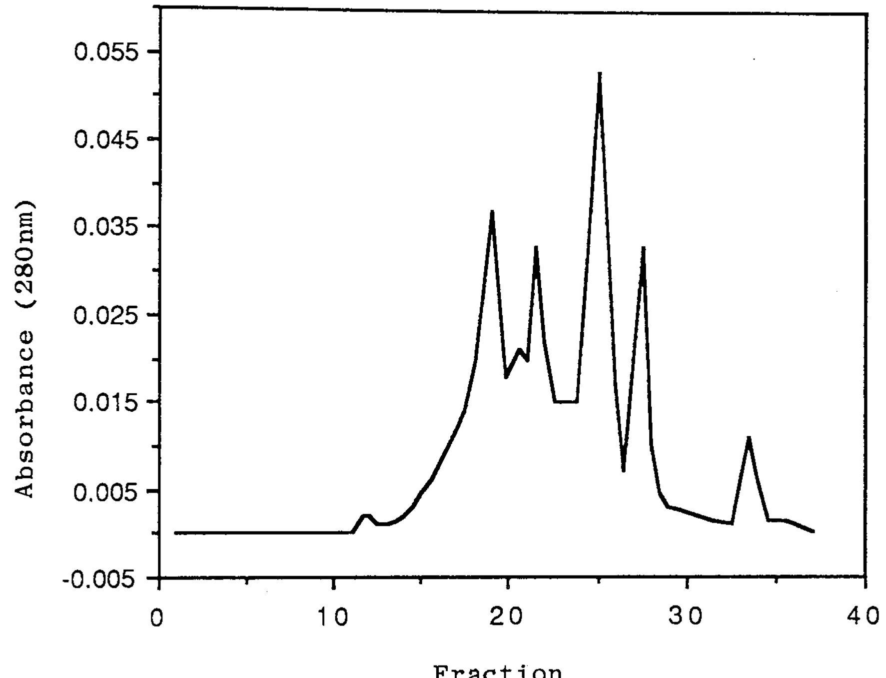


A, Elution profile on Superose 6 of [^{3}H]-labelled material present in incubation medium containing 0.5mM-dithiothreitol, 0.5mM-carbachol and 1mM-dbcAMP in the presence (□) and absence (■) of cells after 1h. In (■) 50,000dpm/ml of D- 6-^{3}H glucosamine was present from the start of the incubation period (3.3.2.2).

B, Absorbance profile at 280nm of medium from an incubation in the presence of cells and secretogogues.

Figure 3.4

Elution profile on Superose 6 of marker compounds.



Marker compounds (molecular mass, kDa) regularly peaked in the following fractions: T_4 coliphage DNA (110,000),11(void volume marker, not shown); thyroglobulin (669), 19/20; apoferritin (443), 22/23; bovine serum albumin (66), 25/26; carbonic anhydrase (29) 28/29; glycyl tyrosine (0.238), 33/34.

second $[^3H]$-labelled peak was of medium molecular mass and much broader than the first. It is henceforth denoted as Peak-2. The apex of Peak-2 was close to the position at which bovine serum albumin eluted from the column. It should be noted that precise molecular masses cannot be assigned to these peaks, since in this system the column was eluted under non-denaturing conditions and the shape of molecules will have affected their elution volume.

A comparison between the elution profiles on Superose 6 of cellular extracts and the incubation medium obtained from secretogogue stimulated cells and unstimulated cells is presented in Fig 3.5. Clearly there is little $[^3H]$-labelled material present in the incubation medium at the beginning of the secretory period compared to the $[^3H]$-labelled material present after 60 min incubation. Furthermore, the elution profiles obtained with cellular extracts before and after incubation with secretogogues are almost identical, and the $[^3H]$-label associated with fractions 10-15 are similar which implies little release of chromatographically equivalent Peak-1 material.

It would seem unlikely that $[^3H]$glucosamine uptake would be solely by mucous epithelial cells, and the medium and low molecular weight material may represent a combination of $[^3H]$-labelled metabolic products from various cell types, or in the case of the peak present in fractions 31-35, unmetabolized radiolabel.

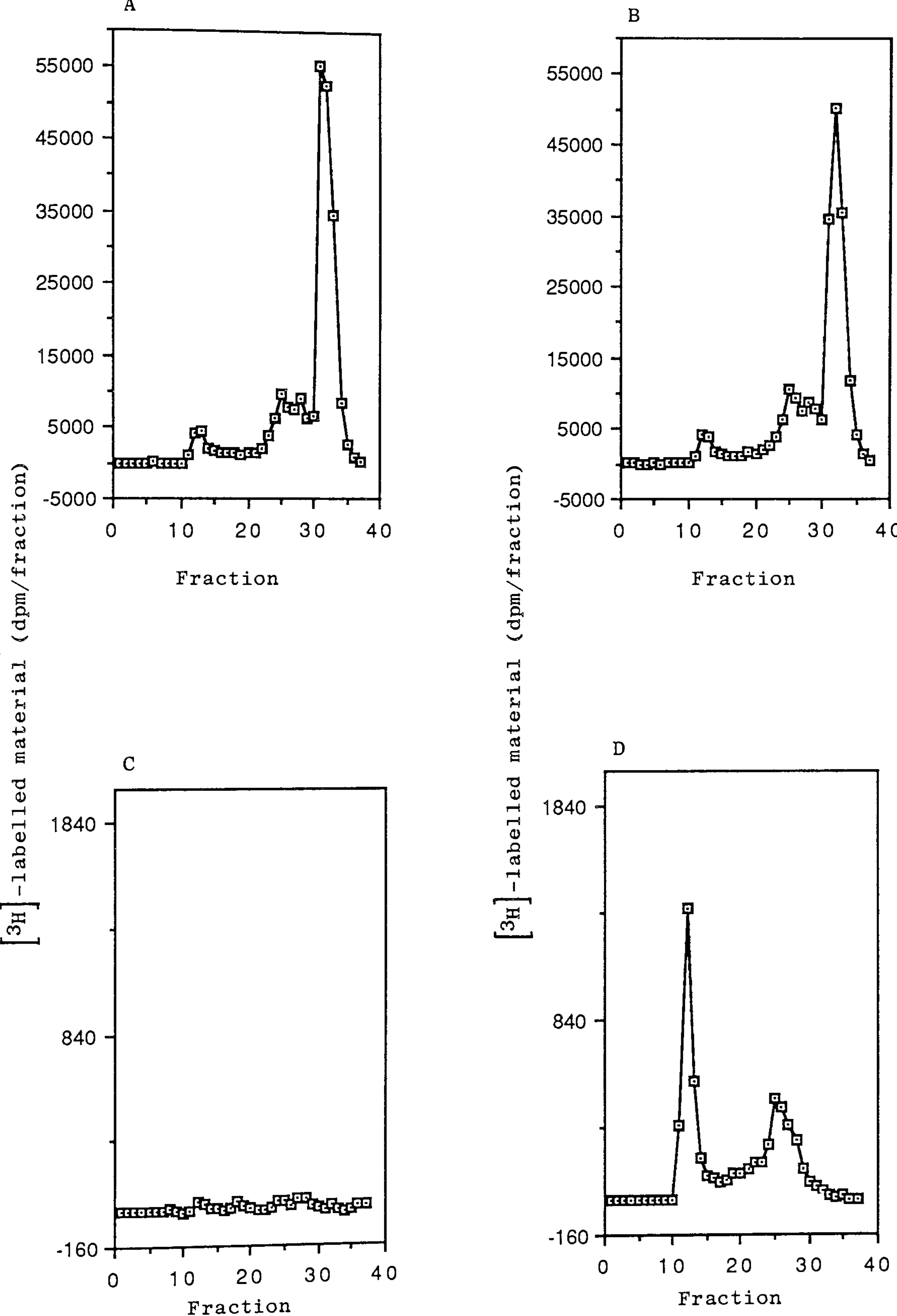
The question of whether the Peak-1 material is mucin is dealt with later (3.3.3). Labelling of bovine serum albumin and intrinsic factor could contribute to the broadly eluting Peak-2 material and this is examined next.

Figure 3.5

Relationship between $[^3H]$-labelled secreted and cellular material obtained from a cell suspension incubated with 0.5mM-carbachol and 1mM-dbcAMP.

Panels A and B represent cellular material present at t=0min and t=60min respectively. Panels C and D represent secreted material present at t=0min and t=60min respectively. Data have been expressed as dpm/fraction.

Figure 3.5



3.3.2.2 Effect of incubation of medium B' and D-[6-^{3}H]glucosamine in the absence of cells

[^{3}H]-labelled material of molecular mass less than 30 KDa present in the incubation medium after 60 min was isolated as the Centricon microconcentrator filtrate. 55228±6581 dpm/ml cell suspension were present (n=9 cell batches). Incubation of [^{3}H]glucosamine (at approximately 50000 dpm/ml) with medium B' in the absence of cells over 60 min gave no [^{3}H]-labelled peaks on analysis of the medium by Superose 6 gel filtration (Fig 3.3 A, filled symbols). Absorbance measurements at 280nm during the gel filtration run (Fig 3.3 B) indicated that bovine serum albumin present in medium B' eluted from the column as a sharp peak in fractions 25 & 26.

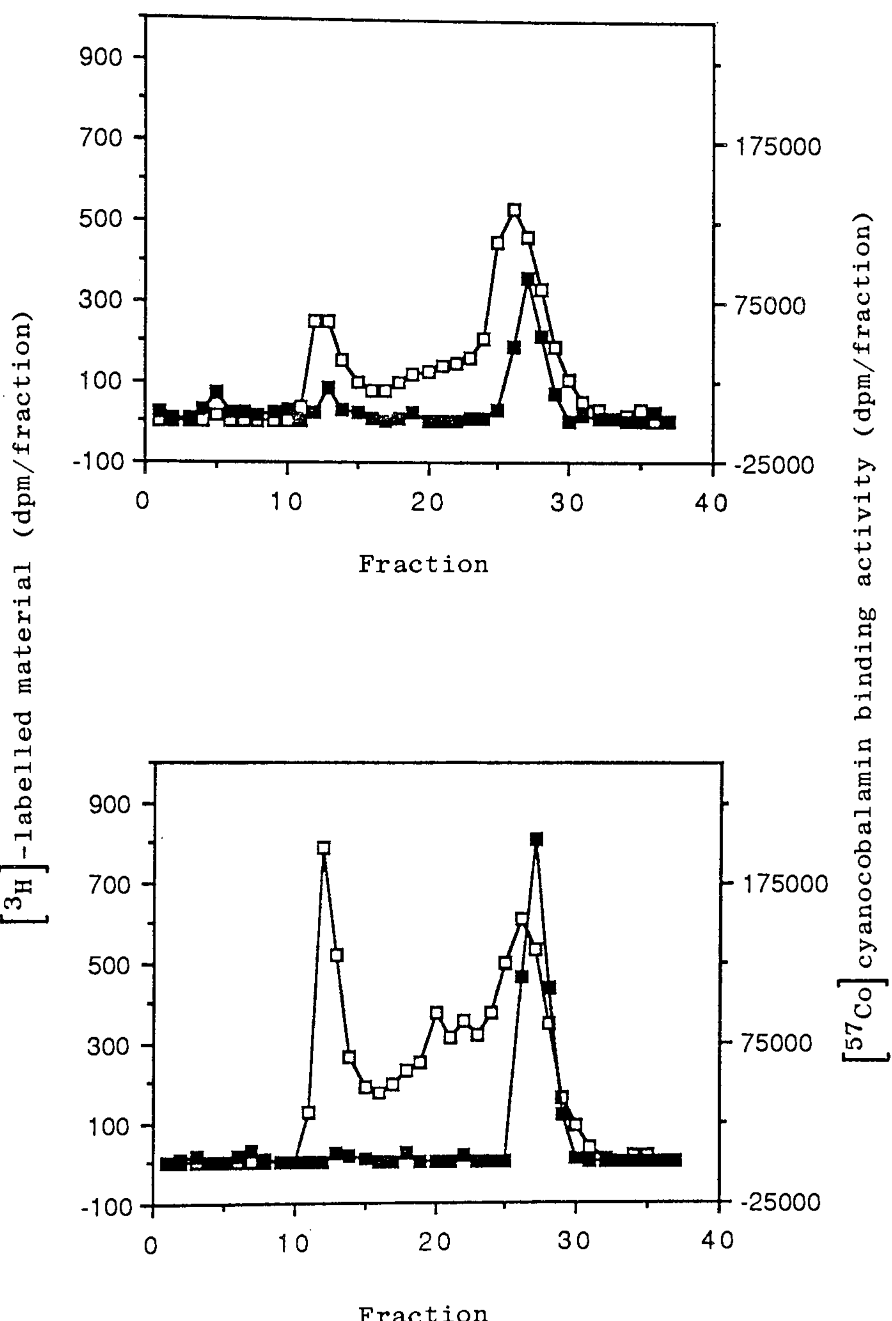
The absence of labelling of the bovine serum albumin peak in the incubation without cells and the lack of identity between the broadly eluting Peak-2 material isolated in the presence of cells and the sharp peak of absorbance at 280nm for bovine serum albumin suggests that Peak-2 is not caused by non-enzymatic labelling of bovine serum albumin.

3.3.2.3 Gel filtration profile of [^{57}Co]cyanocobalamin binding activity

Detection of intrinsic factor in samples of incubation medium fractionated by Superose 6 chromatography was by [^{57}Co]cyanocobalamin binding activity which peaked in fraction 27 (Fig 3.6 A,B). Incubation of cells in the presence of the secretogogue combination carbachol (0.5mM) and dbcAMP (1mM) over 60 min increased [^{57}Co]cyanocobalamin binding activity by 116%.

The elution profile of intrinsic factor on Superose 6 gel filtration is not exactly parallel to that of Peak-2 material suggesting that the latter is not entirely attributable to [^{3}H]-labelling of the

Figure 3.6



Elution profile on Superose 6 of $[^3H]$-labelled material (□ A,B) and $[^{57}Co]$ cyanocobalamin binding activity (■ A,B) released into the incubation medium during incubation of cells for 1h in the absence (panel A) and presence (panel B) of 0.5mM-carbachol and 1mM-dbcAMP.

former. Also the small increase observed in Peak-2 material relative to the large increase in [^{57}Co]cyanocobalamin binding activity in response to secretogogues suggests that any [^{3}H]-labelling of intrinsic factor is probably of low specific activity.

In conclusion, Peak-2 may in part represent [^{3}H]-labelled intrinsic factor, however it would seem likely that this component only represents a small part of this broad peak of material.

3.3.2.4 Effect of incubation time on the viability of non-parietal cells in the presence and absence of secretogogues.

A third explanation for the presence of Peak-2 material is the release of labelled cellular contents during the incubation period. However, incubation for 60 min did not significantly affect non-parietal cell viability (as judged by trypan blue dye exclusion) in either the presence or absence of 0.5mM-carbachol and 1mM-dbcAMP (Table 3.4).

It should be emphasized that this test is not a direct measurement of cell viability but assesses the integrity of the cell membrane of non-parietal cells, which are easily recognizable due to their being smaller than the large parietal cells. The results obtained suggest that no gross loss of cell viability had occurred over the incubation period. The presence of Peak-2 cannot therefore be obviously related to cell breakage and loss of viability.

3.3.2.5 Effect of 0.5mM-dithiothreitol

Addition of 0.5mM-dithiothreitol to the incubation medium increased the size of Peak-1 (fractions 10-15) by 63% when cells were incubated with 0.5mM-carbachol and 1mM-dbcAMP as secretogogues over 60 min (Fig 3.7, see also paired data in Table 4.3), but the effect on Peak-2 material was negligible. In a separate

Table 3.4

Effect of incubation time on the viability* of non-parietal cells in control medium or medium containing 0.5mM-carbachol plus 1mM-dbcAMP

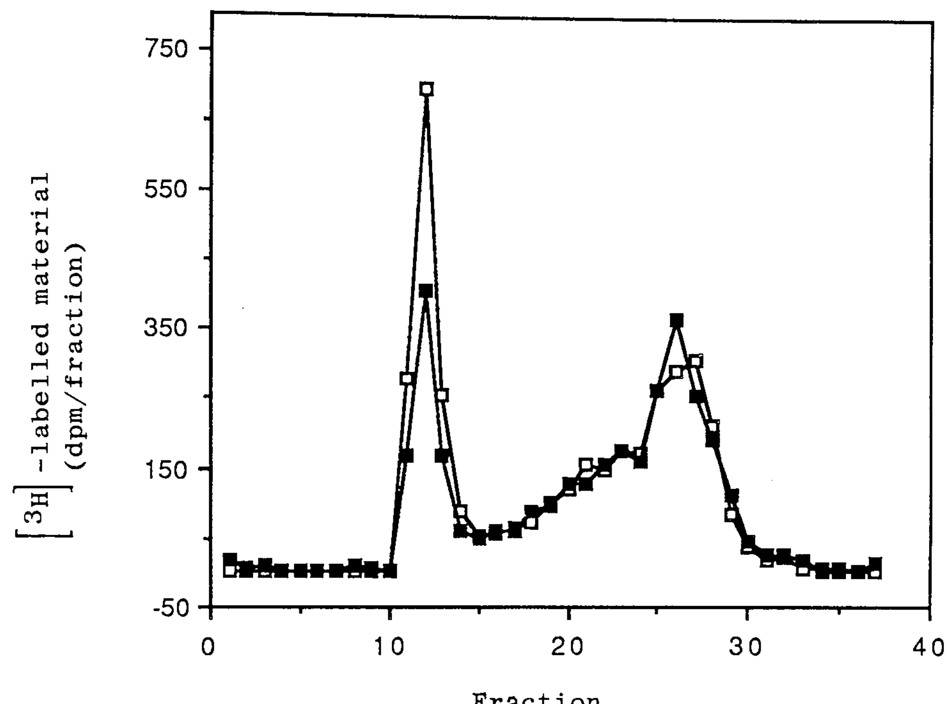
Media	% non-parietal cells excluding trypan blue	
	t=0	t=60
Control	90.2±2.7(3)	89.9±1.2(3)
0.5m-carbachol + 1mM-dbcAMP	91.3±1.8(3)	89.5±3.2(3)

Results are expressed as mean ±SEM with the number of cell batches in parentheses.
There was no significant effect of incubation time on viability in either the presence or absence of secretogogues.

* As judged by trypan blue dye exclusion.

Figure 3.7

The effect of the presence or absence of dithiothreitol in the incubation medium of the isolated cell suspension.



Elution profile on Superose 6 of $[^{3}H]$-labelled material released into the incubation medium containing 0.5mM-carbachol and 1mM-dbcAMP in the absence(■) or presence(□) of 0.5mM-dithiothreitol for 1h.

experiment in the absence of secretogogues Peak-1 was increased by 47% but Peak-2 was unaffected by addition of dithiothreitol.

Methods used to study gastric mucin secretion are hampered by the 'stickiness' of these molecules. Low concentrations of dithiothreitol have been used to disrupt mucus in the preparation of isolated stomach (Payne & Gerber, 1987) and colonic (Roediger & Truelove, 1979) cells. Dithiothreitol (0.5mM) was added to the cell suspension to try to reduce any variability which might arise from released mucin adhering to the cells. Its presence in the incubation medium was observed to prevent the clumping of cells and clearly elevates the material associated with Peak-1.

In conclusion, if Peak-1 is, at least, in part gastric mucin, dithiothreitol may be reducing the adhesiveness of these molecules to mucous epithelial cell membranes and assisting their dispersion into the incubation medium. Since 0.5mM-dithiothreitol increased the size of Peak-1 and made it easier to detect further experiments were generally performed in its presence.

3.3.3 Characterization of [^{3}H]-labelled Peak-1

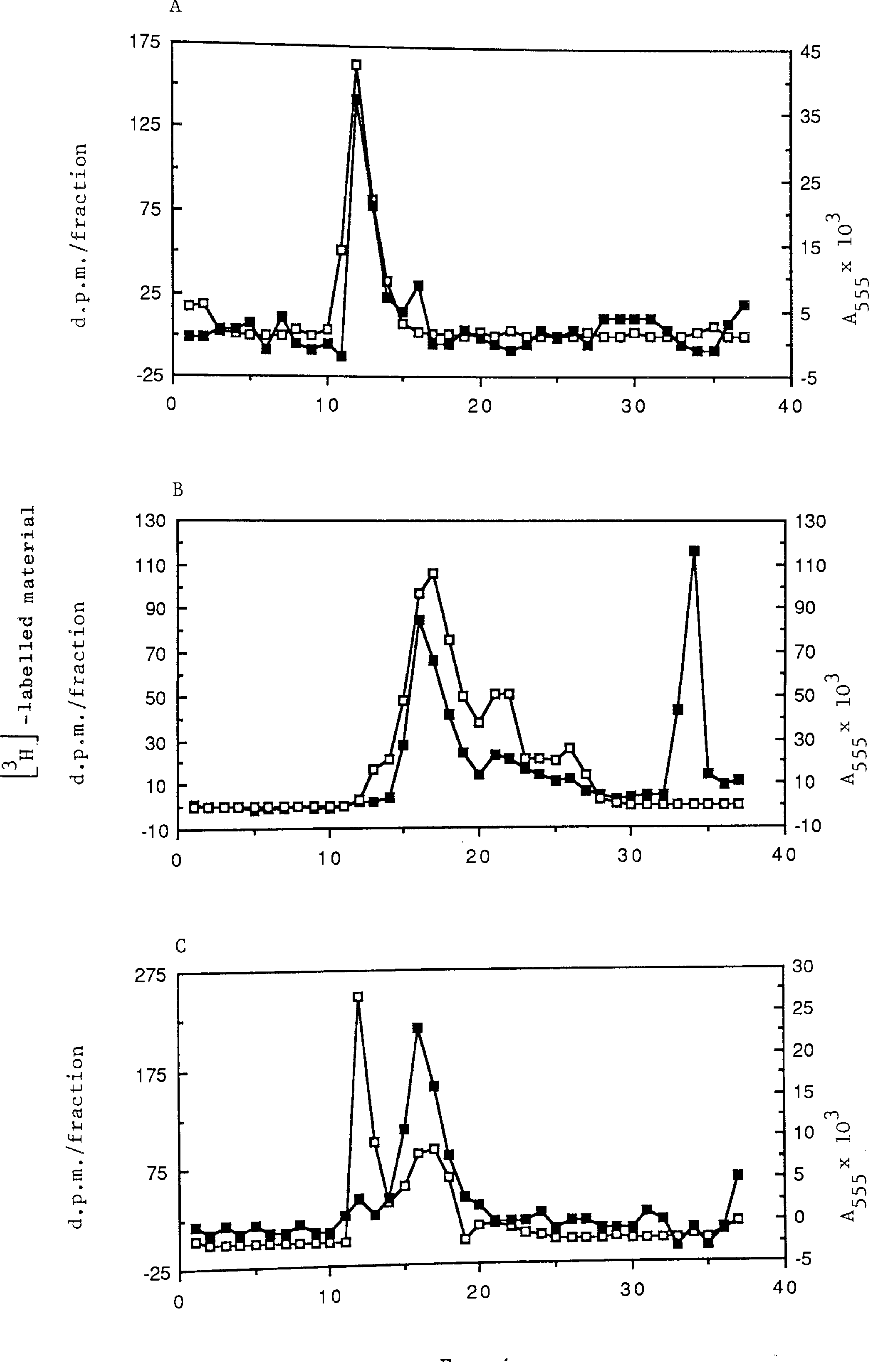
3.3.3.1 Comparison of the gel filtration profiles of purified Peak-1 material and partially purified rat gastric mucin, upon digestion with papain and reduction with 0.1M-dithiothreitol

The elution profile of rechromatographed Peak-1 material isolated from fractions 10-15 was similar to that of partially purified rat gastric mucin on Superose 6 gel filtration (Fig 3.8A). Both materials eluted as sharp peaks close to the void volume of the column between fractions 10-15. Incubation of Peak-1 material with papain for 48 h prior to gel filtration

Figure 3.8

A, Comparison of the elution profiles of purified Peak-1 and of partially purified rat gastric mucin. B, Effect of digestion with papain and C incubation with 0.1M-dithiothreitol on the elution profiles of purified Peak-1 and of partially purified rat gastric mucin. In B, 8% of the initial label appeared in the Centricon 30 filtrate. In all panels, (■) represents unlabelled partially purified rat gastric mucin and (□) represents radioactive Peak-1 material.

Figure 3.8



caused an increase in the elution volume of the main peak (to fractions 15-20), which also contained a shoulder feature (Fig 3.8B, open symbols). This material, nevertheless, showed considerble resistance to complete digestion with papain as the main peak eluted before thyroglobulin, and the majority of the [^{3}H]-labelled (75%) products before apoferritin. Incubation of purified rat gastric mucin with papain produced an elution profile similar to that obtained with the labelled Peak-1 (Fig 3.8B, filled symbols) except a further peak of periodic acid/Schiff staining material was liberated which eluted close to the position at which glycyltyrosine ran. (Fractions 32-35). Incubation of purified gastric mucin with 0.1M-dithiothreitol for 24 hours caused 90% of this material to elute from the Superose 6 column at a higher elution volume (Fig 3.8C, filled symbols). Similar treatment of Peak-1 material caused 52% of this material to elute at a higher elution volume, although to the same position. (Fig 3.8C, open symbols).

The behaviour of purified rat gastric mucin on gel filtration chromatography largely parallels that of purified Peak-1 material. Both materials elute near the column void volume suggesting both are of a similar molecular mass. Proteolytic degradation of mucin is achieved by cleavage of the central protein core in 'naked' or non-glycosylated regions, yielding mucin glycopeptides resistant to further proteolysis. The action of proteases such as pepsin, trypsin, pronase and papain on mucin has been well-studied (Allen <u>et al.</u>, 1984b; Pearson <u>et al.</u>, 1980). The effect of incubation with papain on unlabelled rat mucin is clearly mirrored by Peak-1 material. The majority of the fragments generated have considerable molecular weight and are resistant to further degradation which is consistant with an effect of papain on mucin. The low molecular mass peak obtained on proteolysis of unlabelled gastric mucin may represent loss of carbohydrate residues from mucin during incubation at 60^{0}C, which are unlabelled in Peak-1 material. The

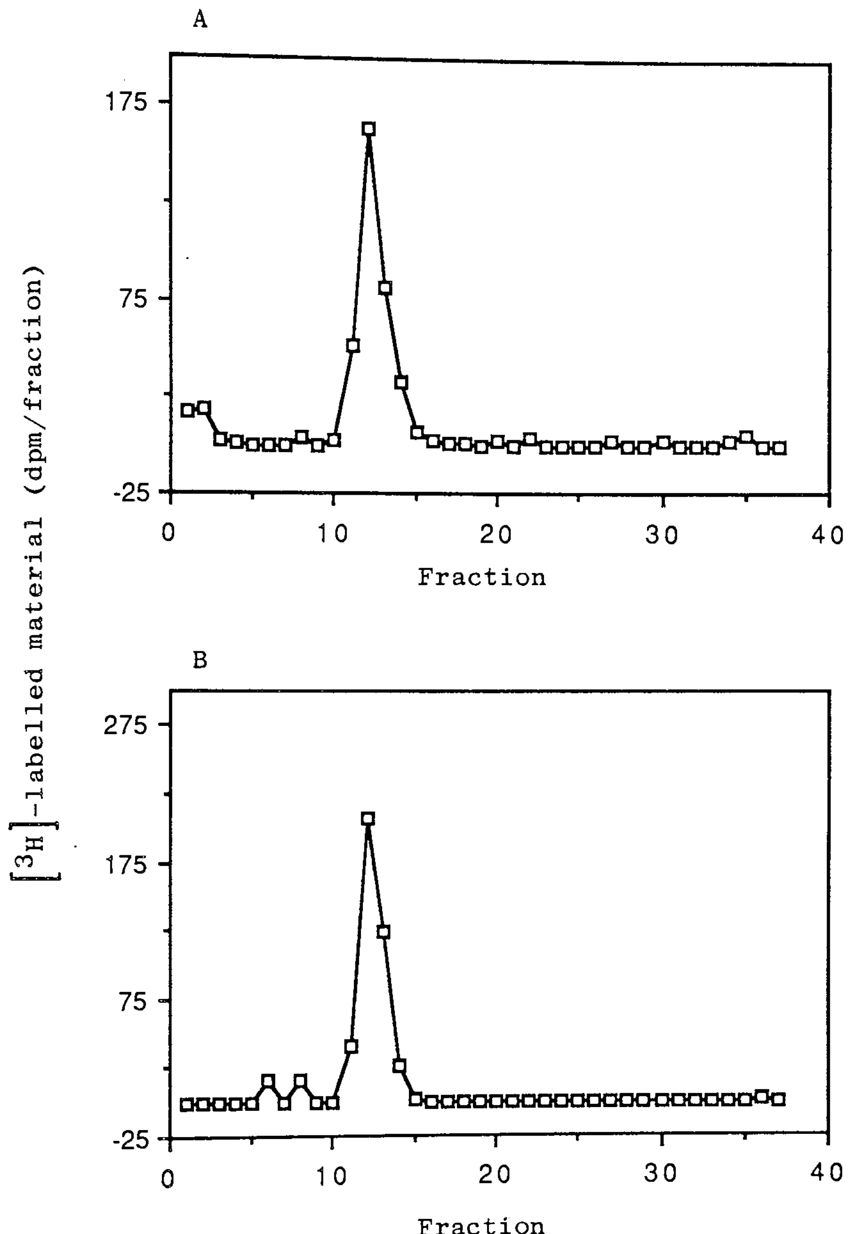
effects of disulphide bond breaking agents such as 2-mercaptoethanol and dithiothreitol on mucin have been widely reported (Pearson et al., 1981; Bell et al., 1985). These chemicals cleave disulphide bonds present in the non-glycosylated regions of the mucin protein core, giving rise to 'subunits', similar in size and properties to mucin glycopeptides. Unlabelled purified rat mucin is almost totally susceptible to dithiothreitol treatment yielding a component with a lower molecular mass. Peak-1 material also showed a reduction in molecular mass, although part of this material was resistant to this treatment. A possible explanation is that although unlabelled mucin scraped from the surface of rat stomachs was homogenized in the presence of protease inhibitors, it had been subjected to proteolysis before homogenization. Carlstedt et al., (1985) have suggested that nicking by proteases may facilitate 'subunit' production upon incubation with thiol agents. Thus mucin secreted by isolated cells may be less susceptible to breakdown by dithiothreitol because it has not been so exposed to proteases. Alternatively as mentioned above the unlabelled rat gastric mucin had been subjected to shear and this could have increased its susceptibility to reduction by dithiothreitol.

3.3.3.2 Effect on hyaluronidase and chondroitinase ABC treatment on the gel filtration profile of Peak-1 material

Incubation of isolated Peak-1 material with either hyaluronidase (Sigma type IX) or chondroitinase ABC over 16 h prior to gel filtration analysis on a Superose 6 column had no effect upon its elution profile when compared to control material incubated in the absence of enzyme. (Fig 3.9 A,B; Fig 3.10 A,B). In all cases [^{3}H]-labelled material eluted close to the void volume between fractions 10 and 15.

Figure 3.9

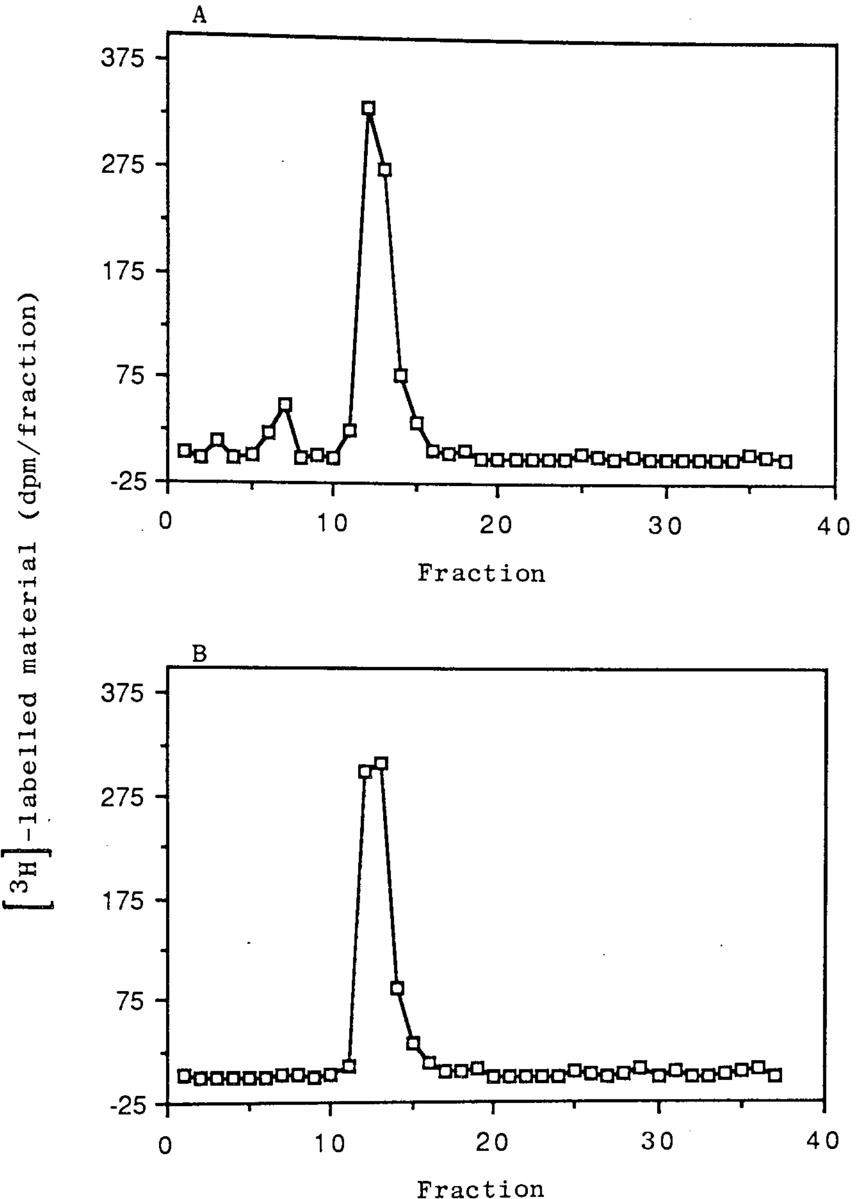
Elution profiles on Superose 6 of purified Peak-1 material preincubated with hyaluronidase.



A, Untreated purified Peak-1 material. B, Hyaluronidase treated purified Peak-1 material. In all panels (□) represents [^{3}H]-labelled Peak-1 material.

Figure 3.10

Elution profile on Superose 6 of purified Peak-1 material preincubated with chondroitinase ABC.



A, Untreated purified Peak-1 material. B, Chondroitinase ABC treated purified Peak-1 material. In all panels (□) represents [^{3}H]-labelled Peak-1 material.

The $[^3H]$-labelled, high molecular mass, material isolated from the incubation medium by gel chromatography could potentially be of proteoglycan origin. Hyaluronidase and chondroitinase ABC are endohexosaminidases capable of destroying the polymeric structure of hyaluronate, chondroitin, chondroitin-4-sulphate, chondroitin-6-sulphate and dermatan sulphate in proteoglycans. Both enzymes produce Δ -4,5-unsaturated disaccharides via a ß elimination reaction and would therefore be expected to reduce the molecular mass of proteoglycans.

In conclusion the lack of effect of hyaluronidase or chondroitinase ABC digestion strongly implies that Peak-1 material is unlikely to be proteoglycan in nature.

3.3.3.3 Caesium chloride density gradient centrifugation of supernatant Peak-1 material, cellular Peak-1 material and unlabelled purified gastric mucin.

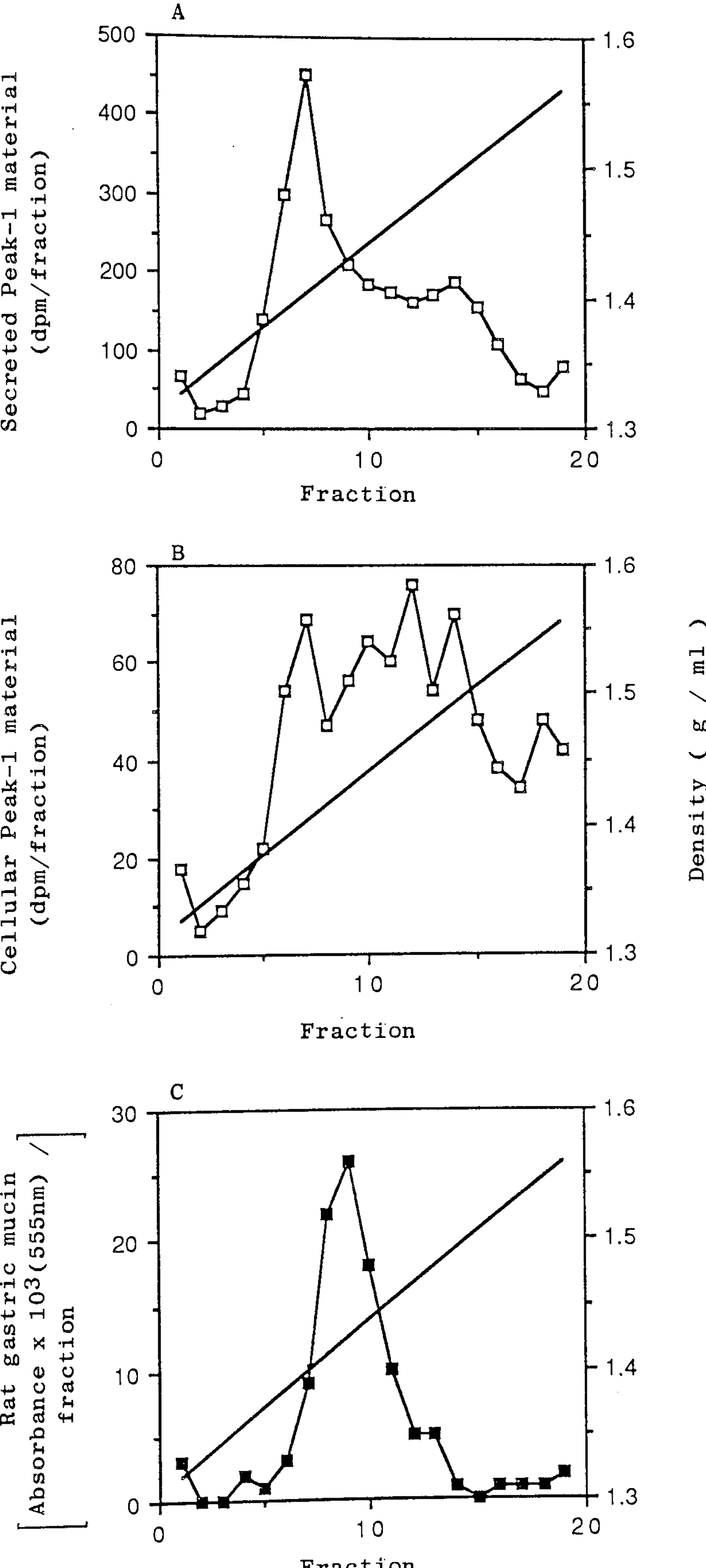
On a caesium chloride density gradient virtually all of the Peak-1 material isolated from samples of incubation medium banded between densities of 1.35 and 1.53g/ml. (Fig 3.11 A), and contained a single well-defined peak at a buoyant density of 1.42g/ml. The majority of Peak-1 material extracted from isolated cells also banded between densities of 1.35 and 1.53g/ml (Fig 3.11 B) although no well-defined peaks were obtained. The behaviour of purified rat gastric mucin on a caesium chloride density gradient was such that periodic acid/Schiff reactive material banded as a single peak between densities of 1.38 and 1.49 g/ml (Fig 3.11 C).

Isopycnic density gradient centrifugation in caesium salts is the method of choice for isolating mucin macromolecules and has been utilized by several investigators (Creeth & Denborough, 1970; Starkey et al., 1984; Hunter et al., 1989). The buoyant density

Figure 3.11

Comparison of the profiles on a caesium chloride density gradient of secreted Peak-1 (A) and cellular Peak-1 (B) material with the profile of partially purified rat gastric mucin (C). The starting density of the caesium chloride solution was 1.45g/ml. In all panels the continuous straight line indicates density.

Figure 3.11



of rat gastric mucin is intermediate between that of protein and DNA, which therefore facilitates effective purification of this material from contaminating substances. The fractionation pattern of Peak-1 material on a caesium chloride density gradient suggests that there was negligible contamination with [^{3}H]-labelled non-glycosylated proteins (which would band at the top of the gradient), or with DNA (which would band at the bottom). However, the distribution of Peak-1 material was broader than that of unlabelled rat gastric mucin and the position of the main peaks of labelled and unlabelled material did not exactly coincide. The fractionation profile of cellular Peak-1 material on caesium chloride density gradient centrifugation displayed distinct heterogeneity and a small amount of labelled material banded near the top of the gradient. This material may possibly represent [^{3}H]-labelled DNA, however, it appears to be only a small fraction (10.9%) of the total labelled material. Differential sulphation may explain the heterogeneity of tissue and secreted Peak-1 material in the present preparation. The profiles of cellular and secreted mucous glycoproteins from rat fundic mucosal segments pre-incubated with [^{35}S] sulphate and [^{3}H]galactose and material scraped from the surface of the rat stomach have been compared using caesium chloride density gradient centrifugation (van Beurden-Lamers et al., 1989). Unlabelled mucin from mucosal scrapings was found to band at 1.46g/ml, whereas labelled mucin was found to run at 1.50g/ml. Inhibition of glycoprotein sulphation by sodium chlorate shifted the density of the labelled mucin nearer to that obtained for unlabelled material. Therefore, the difference in buoyant density was ascribed to the presence of extra sulphate in the labelled material. Sulphation of mucin might explain the presence of the smaller, high density peak in Fig 3.11 A. The presence of mucin precursors may also contribute to the heterogeneity observed for cellular Peak-1 material on the caesium chloride gradient.

3.4 GENERAL DISCUSSION

There is good evidence that Peak-1 material released into the incubation medium of the isolated cell preparation was rat gastric mucin. Firstly, the elution profile of Peak-1 material on a Superose 6 gel filtration column was the same as that of partially purified rat gastric mucin. Secondly, the elution volume of nearly all Peak-1 material and unlabelled purified mucin was increased by pre-treatment with papain, but was resistant to complete digestion. This behaviour is typical of gastric mucous glycoprotein (Pearson et al., 1980). Thirdly the molecular mass of purified gastric mucin can be reduced by reductive cleavage using thiol agents such as 2-mercaptoethanol (Pearson et al., 1980) or dithiothreitol. Peak-1 material also showed such a shift in molecular mass although not all of the material was affected. Fourthly, the fractionation of secreted and cellular Peak-1 material on a caesium chloride density gradient gave buoyant densities typical of gastric mucin (Creeth & Denborough, 1970; Starkey et al., 1974; van Beurden-Lamers, 1989), although the behaviour of unlabelled purified gastric mucin on a caesium chloride density gradient was not exactly analogous. Lastly, Peak-1 material was fully resistant to degradation by hyaluronidase or chondroitinase ABC.

The release of labelled material of lower molecular mass distinct from high molecular mass material has been reported by Roomi et al., (1984) using rat small intestinal slices to study goblet cell mucus secretion, by Slomiany et al., (1987) using cultured scrapings of rat gastric mucosa, by Spohn & McColl, (1987) using human antral mucosa and Seidler et al., (1988) using organ culture of pieces of rabbit gastric mucosa. Recently incorporation of tritiated glucosamine and galactose into mucin and non-mucin components by rat stomach in in vitro organ culture and in vivo has been reported (Hunter et al., 1990). The lower molecular mass non-mucin glycoproteins were found

to be of the same buoyant density as mucin on caesium chloride density gradients and the majority of radiolabel was usually associated with this material. The nature of the lower molecular mass [^{3}H]-labelled material was unidentified, but was suggested to originate from glycoproteins present in the extracellular tissue matrix and/or mucous cell membranes.

It should be emphasized that the specific activities of the labelled peaks eluting from the Superose 6 column are unknown and conclusions about the relative amounts of material making up Peak-1 and Peak-2 cannot be made. The release of Peak-2 material detected in this work may represent membrane glycoproteins released into the incubation medium during exocytosis of mucin granules. However, a close association between Peak-1 and Peak-2 release was not found. Alternatively, the glycoprotein intrinsic factor released from chief cells present in this preparation (Schepp et al., 1983) may contribute in part to Peak-2 material. A further possibility is that Peak-2 may represent glycoproteins released by cellular damage, but measurements of cell membrane integrity using trypan blue dye exclusion did not suggest any gross loss of cell viability. Since the major aim of this work was to investigate [^{3}H]-labelled mucin secretion no further characterization of Peak-2 was attempted.

In conclusion, Peak-1 material isolated by Superose 6 chromatography from an isolated stomach cell suspension radiolabelled with [^{3}H] glucosamine was identified as rat gastric mucin. There are relatively minor differences between this material and that scraped from the surface of the fundic mucosa which may result from modifications to unlabelled material after its secretion in vivo, to modifications (e.g. shear) during its partial purification, or to perturbation of the biosynthetic machinery in the isolated cells.

Nevertheless, this preparation should prove useful in elucidating the factors which control the release of radiolabelled rat gastric mucin.

3.5 SUMMARY

1) Incubation of a suspension of stomach cells with D-[6-^{3}H]glucosamine labelled TCA/PTA precipitable cellular material.

2) [^{3}H]-labelled material of high molecular mass released into the incubation medium of the suspension of stomach cells and analysed by Superose 6 FPLC chromatography (Peak-1) was identified as gastric mucin.

3) [^{3}H]-labelled material of lower molecular mass was also released into the incubation medium.

Chapter Four

FACTORS AFFECTING THE RELEASE OF RADIOLABELLED MUCIN FROM RAT ISOLATED CELLS.

4.1 INTRODUCTION

The main objective of this chapter was to investigate the actions of some potential secretogogues on mucin release in vitro using the [^{3}H]-labelling/gel filtration methodology described in Chapter 3.

4.1.1 Potential mucin secretogogues used in this section

Release of peak-1 material from the rat isolated stomach cell preparation was examined in the presence of secretin, epidermal growth factor, isoprenaline and the main acid secretogues histamine, carbachol (a muscarinic cholinergic agonist) and gastrin. Some properties of these agents are outlined in Table 4.1. Where known agents were used at concentrations which had been previously reported to stimulate/inhibit uptake of the weak base aminopyrine (an index of acid secretory activity) in gastric parietal cells.

Effects of prostaglandins on secretion of [^{14}C]-labelled high molecular weight material in vitro by pieces of rabbit gastric mucosa preincubated with [^{14}C]N-acetylglucosamine in organ culture were presented recently by Seidler et al., (1988). 10^{-6}M-prostaglandin E_2 increased release of label by approximately 50% above control and 10^{-5}M-prostaglandin $F_{2\alpha}$ induced at 30% stimulation. To prevent duplication of results, an investigation of the effects of prostaglandins on the release of Peak-1 material was not attempted here.

The time-dependance of Peak-1 release was assessed in the presence and absence of carbachol plus dbcAMP. This combination of secretogogues was used with the intention of maximally stimulating the cells. Dibutyryl cyclic AMP should activate any secretory pathway involving cyclic AMP-dependent protein kinase

Table 4.1

Some properties and reasons for use of agents investigated for effects upon mucin release using the D-[6-^{3}H]glucosamine labelling protocol

Agent	Structure	Molecular mass (Da)	Reason for investigation
Secretin	Polypeptide of 27 amino acids	3055	Reported to be gastric mucin secretogogue in vivo (Neutra & Forstner, 1987)
Isoprenaline	$(HO)_2C_6H_3-CH(OH)-CH_2-NH-CH(CH_3)_2$	215	Stimulates isolated gastric parietal cells to secrete acid (Rosenfeld, 1984)
Carbachol	$CH_3-N^{+}(CH_3)_2-CH_2-CH_2-O-C(=O)-NH_2$	183	Well-established acid secretogogue. Mucin secretogogue in vivo (Neutra & Forstner, 1987)
Gastrin-17	Polypeptide of 17 amino acids	2080	Gastric acid secretogogue (Soll & Berglindh, 1987)

Table 4.1 con't

Agent	Structure	Molecular mass (Da)	Reason for investigation
Histamine	(imidazole ring, HN, N)—CH_2—CH_2—NH_2	111	Gastric acid secretogogue (Soll & Berglindh, 1987)
EGF	Polypeptide of 53 amino acids	6045	Involved in maintenance of gastrointestinal mucosa and protection from damage (Kontarek, 1988)

while carbachol, probably acting through calcium-mediated processes is known to be a secretogogue *in vivo* (1.4.2.2).

4.2 METHODOLOGY

4.2.1 Incubation procedures

The concentration of stock solutions of potential secretogogues, the solvent and the volume added to polyethylene scintillation vials used for incubation were as shown in Table 4.2. An equivalent amount of saline was added to control vials. Cell suspension (at 10^7 cells/ml) was added to vials to give a total volume of 2ml. The air space in the vials was gassed with 95% O_2/5% CO_2 and incubation was for 45 min at 37^0C with shaking at 150 cycles/min, except in the time course experiments where incubation of vials was terminated at 15, 30, 45 and 60 min.

4.2.2 Protocol for the purification of experimental samples

[^{3}H]-labelled material present in the incubation medium after the secretory period and [^{3}H]-labelled cellular material were prepared for Superose 6 gel filtration as described in 3.2.3.1 and 3.2.3.2. In experiments investigating potential secretogogues, 12 samples of incubation medium (6 treatments in duplicate) and 1 sample of cellular material were usually chromatographed over a three - day period. A typical experiment of this type required one week to complete and is illustrated in Fig 4.1.

4.2.2.1 Operation of the Superose 6 column

The buffer used to equilibrate and elute samples from the column was as described in 3.2.3.3. In all experiments the flow rate was 0.5ml/min and fractions were collected every 1.2 min. [^{3}H]-labelled material in the fractions was detected by scintillation counting (see 2.2.2) using Optiphase Safe (Pharmacia-LKB, Milton Keynes).

Table 4.2

Potential secretogogues added to the isolated cell suspension

Agent	Concentration of stock solution	Solvent	Volume added to incubation vial
0.5mM carbachol + 1mM dbcAMP*	-	-	-
Secretin (10^{-7}M)	$2x10^{-5}$M	saline	10µl
Isoprenaline (10^{-5}M)	$2x10^{-3}$M	saline	10µl
Carbachol (0.5mM)	0.1M	saline	10µl
Gastrin (10^{-7})	$2x10^{-5}$M	saline	10µl
Histamine (0.5mM)	0.1M	saline	10µl
Epidermal growth factor ($2x10^{-7}$M)	$3.2x10^{-5}$M	saline	12.5µl

* These agents were added directly to the resuspension medium and not to th incubation vials.

Figure 4.1

Experimental protocol

The protocol below describes an experiment involving 12 samples of incubation medium and 1 sample of cellular material. Control samples were always sample numbers 1 and 2. Secretogogue treatments were always added to sequential vials i.e. 3 and 4, 5 and 6 etc.

MONDAY	TUESDAY	WEDNESDAY	THURSDAY	FRIDAY
Collate radioactivity data from previous experiment	Isolate & incubate cells. Store samples	Chromatograph samples 1 3	Chromatograph samples 11 12	Chromatograph samples 4 2
Prepare for experiment	Equilibrate Superose 6 column with buffer overnight	5 7 9	10 8 6	cellular samples Equilibrate Superose 6 column with azide/water Count radioactivity over weekend

Between samples the column was flushed for 30 min with buffer to ensure complete elution of previously chromatographed $[^3H]$-labelled material. This was checked by counting fraction seven (which elutes before the void volume) for each run.

4.2.3 Presentation of data

The size of Peak-1 was estimated by adding together the dpm in fractions 10-15. Very similar results were obtained if the area of Peak-1 was estimated from width at half peak height x peak height. Since the cell concentration in all experiments was 10^7 cells/ml data could be expressed as dpm released/ml cell suspension. The effect of an agent upon the Peak-1 material in the incubation medium could also be expressed by normalizing the data to % stimulation above basal. This was calculated by the following expression:-

$$\text{\% stimulation above basal} = \frac{\left(\begin{array}{c}\text{dpm/ml cell suspension in presence of agent} - \text{dpm/ml cell suspension in absence of agent}\end{array}\right)}{\text{dpm/ml cell suspension in absence of agent}} \times 100$$

Normalization of the data enabled comparisons between sets of data despite variations in the absolute magnitude of the untransformed data. Control and experimental observations were always made on the same cell batch. Where possible the computer program FIT (Barlow, 1983) was utilized to estimate the half-maximally effective concentration (EC_{50}) of an agent. Since not all data in this thesis could be fitted using this procedure both fitted and 'line' plots are presented.

4.3 RESULTS AND DISCUSSION

4.3.1 Quantitation of Peak-1

Recovery of [^{3}H]-labelled material in the incubation medium, from Centricon 30 microconcentrator retentates and filtrates was 99±1% (n=4) (see 3.2.3.1 for methodology). In addition, in experiments of the type illustrated in Fig 4.1 there was no significant loss (paired t-test) of Peak-1 material during 3 days of storage at 4^{0}C. Thus, control release of Peak-1 material in the initial eight experiments of this type was 703±80 dpm/ml cell suspension in the sample measured on day one and 667±83 dpm/ml cell suspension in the duplicate sample measured on day three. Recovery of purified Peak-1 material re-run on the Superose 6 column was 68±2% (n=10). Recovery of total counts from chromatographed cellular extracts was 72±2% (n=3).

Complete recovery of [^{3}H]-labelled material after processing in Centricon 30 microconcentrators indicates negligible interaction of the radiolabelled material released into the incubation medium with the hydrophilic ultrafiltration membrane, or with the plastic components of the filtration unit. No loss of Peak-1 material during approximately two days of storage suggests that proteolytic or bacterial breakdown of [^{3}H]-labelled mucin was negligible during storage in the microconcentrators. The recovery of Peak-1 material or total counts from the Superose 6 column was approximately 70% for samples of incubation medium and cellular extracts. The remaining material may have adsorbed to the column during the run possibly to the filter at the top of the column. Indeed deposition of material on the top filter gave rise to increased column backpressure and the filter required replacing every two weeks. Nevertheless the repeatable recovery of [^{3}H]-labelled material from the column implies that accurate quantative measurements are possible with this system.

4.3.2 Time-dependance of release of [^{3}H]-labelled gastric mucin

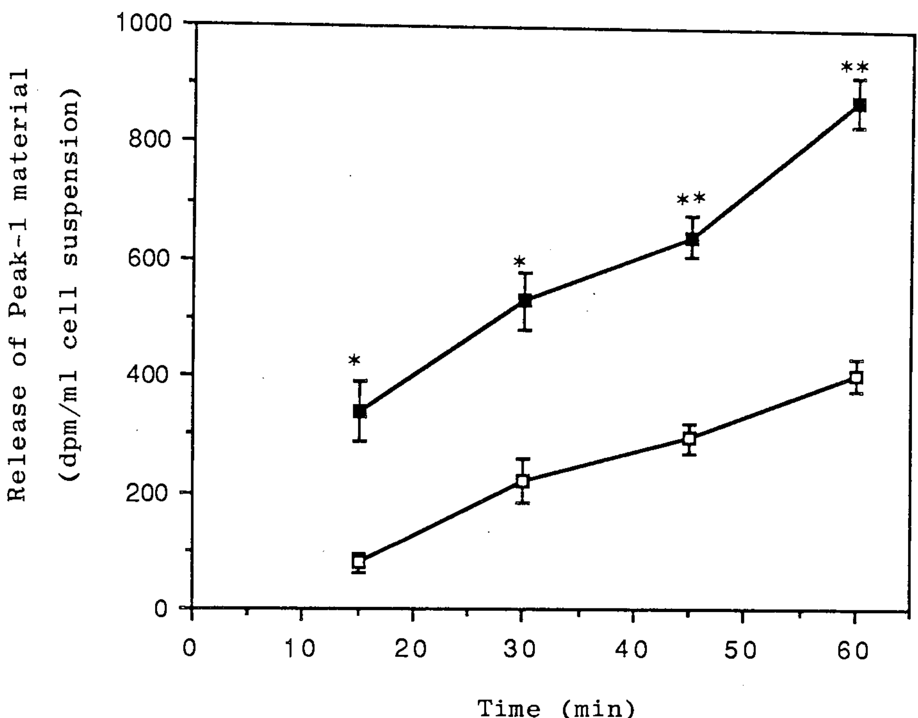
Release of [^{3}H]-labelled gastric mucin into the incubation medium was linearly related to time ($P<0.01$ by regression analysis) for cells incubated in the presence or absence of 0.5mM-carbachol and 1mM-dbcAMP (Fig 4.2). The rate of release of gastric mucin from stimulated cells over 15-60 min was 63.3% greater than that from non-stimulated cells, as determined by the increase in regression coefficient (Fig 4.2). At each time point tested, significantly more material was released by cells incubated in the presence of 0.5mM-carbachol and 1mM-dbcAMP than in their absence (Fig 4.2).

Linear release of proteins pulse/chase labelled with [^{14}C]N-acetyl-D-glucosamine has been reported by Seidler et al., (1988) using rabbit mucosal explants. In this system steady release was obtained for 13h, however degenerative changes in fundic explants were observed after 16h of incubation. An effect of 0.5mM-carbachol and 1mM dbcAMP on mucin release was observed after 15 min incubation with these agents in the isolated cell preparation. Studies on the biosynthesis and processing of rat gastric mucin (Jentjens et al., 1986) suggest that at least 60 min is required for mucin labelled with [^{35}S]cysteine to be secreted from stomach segments. It is therefore likely that the effects of carbachol and dbcAMP were mediated through changes in mucin secretion rather than changes in synthesis.

The possibility that radiolabelled mucin does not adequately represent bulk mucin has to be considered. Further discussion of the physiological significance of the time-course of [^{3}H]-labelled mucin release will be presented in 6.3.1.4, where the present data and that for bulk mucin release (measured by solid phase enzyme immunoassay) will be compared and discussed together.

Figure 4.2

The effect of time on the release of $[^3H]$-labelled gastric mucin into the incubation medium.



Results are presented as means ± S.E.M. from three cell batches. (□), control ; (■), 0.5mM-carbachol plus 1mM-dbcAMP. Material released from control cells in 60min was 5.7 ± 1.4% of chromatographically equivalent cellular material. The equation for the control regression line is $y= -14+7.033x$ and that for the secretogogue combination is $y=166.5+11.468x$. The effect of 0.5mM-carbachol and 1mM-dbcAMP at each time point was assessed by paired t-test, $*P<0.025$; $**P<0.01$. Results have been corrected for material present at the start of incubation which was 168 ± 17 dpm/ml cell suspension.

4.3.3 Relationship of released material to cellular material

An alternative way of presenting the results was to express release into the medium as a percentage of chromatographically equivalent cellular material. Thus release of mucin after 60 min of incubation was 6.4±1.2% and 12.9±3.1% of cellular material in the absence and presence of 0.5mM-carbachol and 1mM-dbcAMP respectively (n=4; $P<0.05$ for the effect of agents by a paired t-test). Release of peak-2 labelled material was 4.5±0.4% and 6.0±0.7% of chromatographically equivalent cellular material in the absence and presence of 0.5mM-carbachol and 1mM-dbcAMP (no significant difference by paired t-test). Release of [^{14}C] N-acetyl glucosamine labelled glycoproteins predominantly of high molecular mass has been reported by Seidler et al., (1988) using rabbit gastric fundic explants. In this system approximately 9% of the cellular material was released into the culture medium after 16h of incubation under control conditions. Stimulation of glycoprotein secretion with 10μM-16,16-dimethylprostaglandin E_2 over the same period significantly increased release of cellular material to approximately 12%. These results are similar to those obtained with the isolated cell preparation in that stimulation of secretion above basal levels releases only a small proportion of cellular labelled material, and implies that secretogogues modulate a slow continuous secretion of mucin.

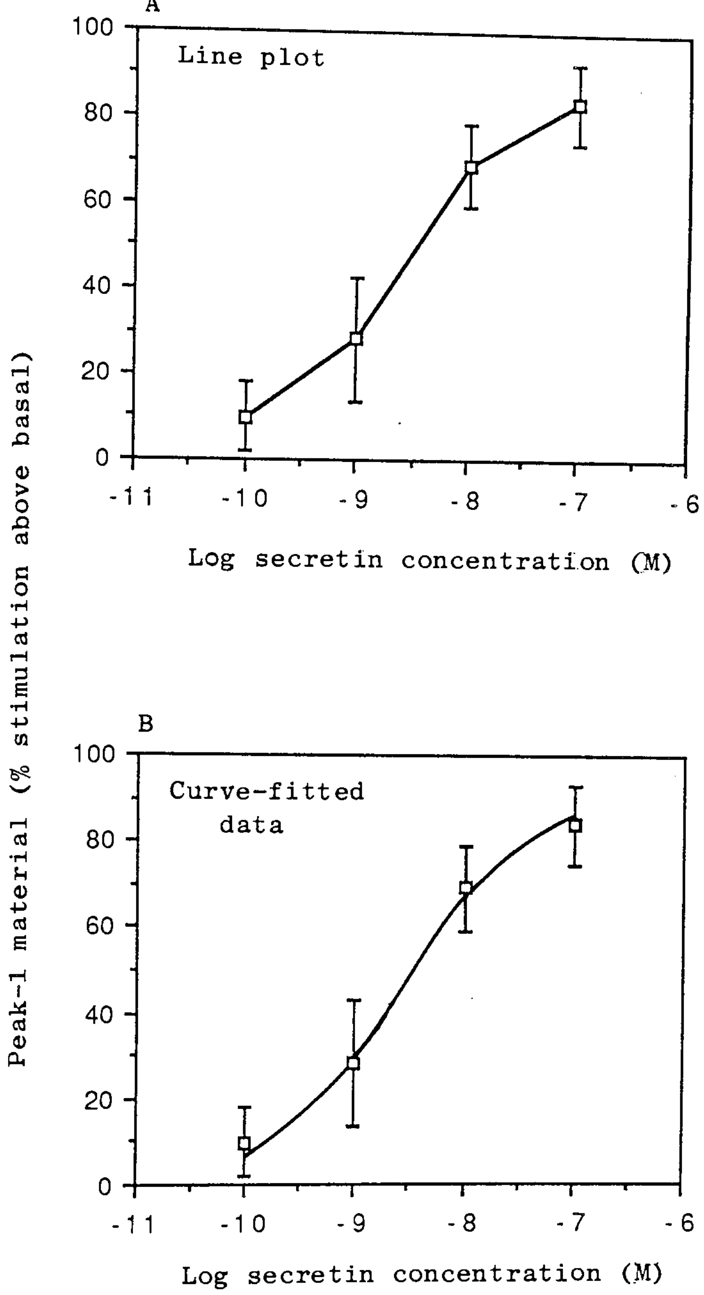
4.3.4 Effects of concentration of secretin and isoprenaline on the release of Peak-1 material

4.3.4.1 Effect of 0.1nM-100nM secretin

Increasing the secretin concentration from 0.1nM-100nM caused a dose-dependent ($p<0.01$ by analysis of variance) increase in the [^{3}H]-labelled mucin present in the medium after a 45 min incubation (Fig 4.3). The half-maximally effective concentration of secretin was

Figure 4.3

Effect of concentration of secretin on $[^3H]$-labelled mucin in the incubation medium.



Data are presented as means ± S.E.M. from four cell batches. 650 ± 79 dpm/ml was present after 45min in the control cell suspension. This represented 8.5 ± 1.4% of chromatographically equivalent material present in cells at the start of incubation.

determined by the computer program FIT to be 2.3nM, and the stimulation at 100nM secretin was 84% above the basal release.

4.3.4.2 Effect of 1nM-10μM isoprenaline

A dose-dependent ($p<0.01$ by analysis of variance) increase in the peak-1 material in the incubation medium after 45 min was found when the isoprenaline concentration was increased from 1nM-10μM (Fig 4.4). Stimulation above basal was 33% at 1μM isoprenaline and the half-maximally effective concentration of isoprenaline as determined by the computer program FIT was 34nM.

Discussion of the release of [^{3}H]-labelled rat gastric mucin induced by secretin and isoprenaline will be presented in Chapter 6 along with data obtained with these agents using an indirect competitive solid-phase enzyme immunoassay to measure total mucin release. A comparison of the results obtained with each method will be presented in the General Discussion (Chapter 7).

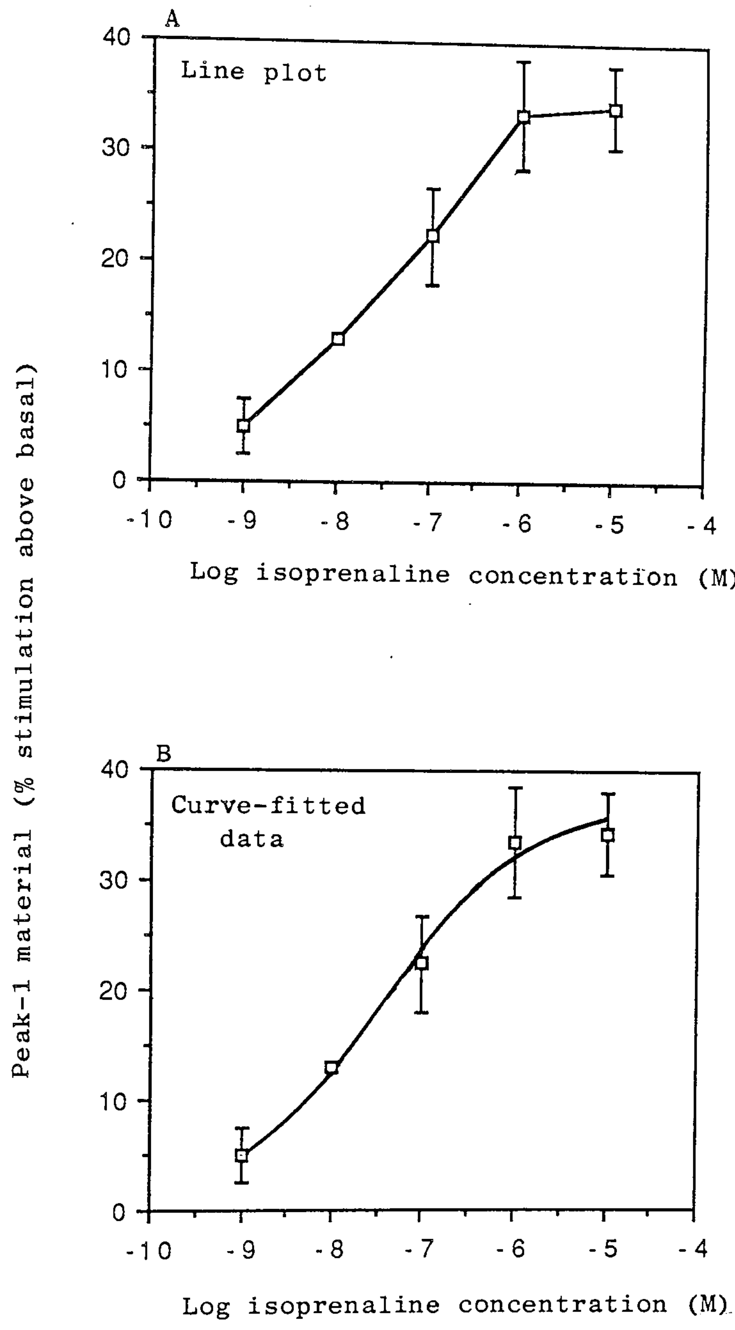
4.3.5 Effects of acid secretogogues; carbachol, gastrin and histamine on the release of Peak-1 material

4.3.5.1 Effect of 0.5mM-carbachol

Release of mucin was significantly stimulated ($p<0.001$; Table 4.3) by 0.5mM-carbachol in the presence of 0.5mM-dithiothreitol over a 45 min incubation period. Peak-1 material was 18±3% (n=6) above basal after 45 min of incubation. Removal of dithiothreitol from the incubation medium significantly ($p<0.01$, by paired t-test) reduced the radioactivity in Peak-1 when cells were incubated in the presence or absence of 0.5mM-carbachol. Under these conditions the

Figure 4.4

Effect of concentration of isoprenaline on the release of [^{3}H]-labelled mucin.



Data are presented as means ± S.E.M. from four cell batches. 500 ± 74 dpm/ml was present after 45min in the control cell suspension. This represented 5.9 ± 0.75% of chromatographically equivalent material present in the cells at the start of incubation.

Table 4.3

Effect of agents on release of [^{3}H]-labelled mucin from isolated stomach cells

Agent	Dithiothreitol (0.5mM)	n	Peak-1 material (dpm/ml cell suspension)	
			Control	+ Agent
Carbachol (0.5mM)	-	6	397±25	509±19***
Carbachol (0.5mM)	+	6	546±39	643±43***
Gastrin (100nM)	+	4	517±40	510±44
Histamine (0.5mM)	+	7	651±71	632±90
EGF (200nM)	-	4	750±114	740±114

Results are presented as means ±SEM from n cell batches. The incubation time was 45 min. The Peak-1 material in the incubation medium in control incubations ranged from 3.9 to 9.0% of chromatographically equivalent cellular material in experiments without dithiothreitol and from 5.0 to 11.9% when dithiothreitol was present. ***, $P<0.001$ for the effect of agent by paired t-test.

stimulatory effect of carbachol was 29±4% (n=6) above the control cells but was not significantly altered (paired t-test).

Most experiments were performed in the presence of 0.5mM dithiothreitol since this agent increased the size of Peak-1 and made it easier to detect. An explanation for the action of dithiothreitol was presented in 3.3.2.5. A further possibility is that dithiothreitol could act as a mucous secretogogue. If dithiothreitol were acting as a secretogogue the cells could not be maximally stimulated since no response to other secretogogues would be evident. In addition the stimulatory effect of carbachol was not significantly altered by the addition of dithiothreitol to the incubation medium. However, an exact duplication of results in the absence and presence of dithiothreitol was not obtained and the possibility that dithiothreitol may modify the responsiveness of cells to other secretogogues cannot be ruled out.

Further discussion of the implications of the data obtained with 0.5mM-carbachol will be presented in Chapter 6 together with measurements of bulk mucin secretion in the presence of this agent.

4.3.5.2 Effect of 100nM-gastrin and 0.5mM-histamine

Incubation of cells with 100nM-gastrin produced no effect on the Peak-1 material present after a 45 min incubation period (Table 4.3). Similarly, incubation of the cells in the presence of 0.5mM-histamine over 45 min did not elicit a secretory response above basal levels (Table 4.3).

There is good evidence that both gastrin and histamine are important gastric acid secretogogues (see Soll & Berglindh, 1987, for review). The concentrations of gastrin and histamine used in these

experiments were chosen because they have been shown to elicit near maximal acid secretion from canine parietal cells (Soll & Berglindh, 1987).

Although gastrin and histamine have been shown to increase gastric mucin biosynthesis in a dose-dependent manner in a canine gastric mucous cell culture system (Boland et al., 1986), results obtained in this section of work imply that both gastrin and histamine are not mucin secretogogues in the rat isolated cell preparation. The normal polarized nature of the cells in the epithelium is lost in isolated cell preparations and damage to the secretory machinery and receptors during isolation could also explain the lack of effect of histamine and gastrin. However, damage to any potential histamine H_2 receptor on mucous cells seems unlikely as parietal cells in the preparation exhibit a functional histamine H_2 receptor. Intravenous infusion of gastrin (at 32µg Kg^{-1} h^{-1}) and histamine (0.32mg Kg^{-1} h^{-1}) in the cat both increased the presence of sugars in the gastric juice in vivo (Vagne & Perret, 1976). Such data could have arisen from the release of mucin or its increased degradation concomitant with increased acid secretion.

In conclusion it seems likely that neither gastrin nor histamine act directly upon mucous epithelial cells and therefore these agents do not act as mucin secretogogues in this system.

4.3.5.3 Effect of 200nM-epidermal growth factor (EGF)

200nM-EGF did not significantly alter the Peak-1 material in the medium after a 45 min incubation period. (Table 4.3). Experiments with EGF were performed in the absence of 0.5mM-dithiothreitol to ensure that the structure and therefore the biological activity of EGF was not disrupted (EGF contains 3 disulphide bridges). When 0.5mM dithiothreitol was included in the incubation medium Peak-1 material was 825dpm/ml cell suspension after incubation for 45 min

in the presence of 200nM-EGF, and 925 dpm/ml cell suspension in the absence of this agent (single experiment). There was therefore no obvious response to EGF if dithiothreitol was present.

Yoshida et al., (1987) claimed that human biosynthetic EGF (at 10ng/ml) stimulated [^{3}H]-labelled mucin release by primary cultures of rabbit fundic mucosal cells incubated with [^{3}H]glucosamine. However it seems likely that not all of the [^{3}H]-labelled material released from the cells was mucin since only 40% of the total radioactivity was assosciated with periodic acid/Schiff reactive material when medium was subjected to SDS-PAGE analysis. In addition the effects of EGF on release of labelled glycoproteins was not dissociated from potential effects of EGF on their biosynthesis.

In conclusion, pulse/chase labelling of the isolated cell preparation with D-[6-^{3}H]glucosamine followed by the quantitation of radiolabelled mucin has shown that it is possible to measure mucin release from cells in suspension in vitro and has led to the identification of secretin, isoprenaline and carbachol as mucin secretogogues in this system.

4.4 SUMMARY

1) Effects of secretogogues on mucin release can be detected using a suspension of isolated cells from rat stomach in vitro.

2) Release of mucin in the presence or absence of 0.5mM-carbachol and 1mM-dbcAMP was essentially linearly related to time. After the onset of incubation stimulated cells released only 12.9% of chromatographically equivalent cellular material in 60 min. These results suggest that secretagogues modulate a slow continuous mucin secretion.

3) Secretin and isoprenaline released $[^3H]$-labelled mucin in a dose-dependent manner. The low EC_{50} displayed by secretin suggests that this agent may be of physiological importance. No reports that isoprenaline stimulated mucin release had appeared previously.

4) Of the acid secretogogues, carbachol, histamine and gastrin, only carbachol was capable of significantly enhancing the release of radiolabelled rat gastric mucin. EGF was also ineffective.

Chapter Five

DEVELOPMENT OF AN INDIRECT COMPETITIVE SOLID-PHASE ENZYME IMMUNOASSAY TO QUANTITATE SECRETION OF RAT GASTRIC MUCIN.

5.1 INTRODUCTION

Immunochemical assays are a potential means of assessing bulk mucin release and of obviating the requirement for sample purification prior to assay. This section of work describes the development of two solid phase enzyme immunoassays capable of quantitating secreted rat gastric mucin.

Some terminology commonly used in immunological and enzyme immunoassay procedures is presented in Table 5.1.

5.1.1 Enzyme immunoassays

Enzyme immunoassays (EIA) are founded upon two important biological phenomena. The first is the ability of the vertebrate immune system to produce proteins (antibodies) with a affinity for a particular foreign compound (antigen or hapten). The second is the extraordinarily high catalytic power of certain enzymes, which enables their presence to be easily detectable. The reaction between the immunoreactants (antibody and antigen) and its subsequent detection using enzymes (usually conjugated to secondary antibodies) as indicators form the two main steps of EIA procedures.

Antigen/antibody complexes may also be detected by using radioactive isotopes to replace the enzyme label. However, this is generally less satisfactory than using an enzyme label for the following reasons; (i) the isotopes used as tracers often have a short half-life, and (ii) the isotopes may represent a health hazard and require special disposal procedures. The major advantages and disadvantages of EIA are summarized in Table 5.2.

Table 5.1

Glossary of immunological terms used in this work

Term	Definition
Adjuvant	A substance that increases the biosynthesis of antibody in response to antigen
Affinity	The intrinsic binding power of an antibody for an antigen
Antibody (immunoglobulin)	A protein capable of specific combination with an antigen
Antigen	Any foreign substance that elicits an immune response
Antigenic determinant (epitope)	A site on an antigen to which an antibody specificially binds.
Antiserum	A serum containing antibodies against a specific antigen
Detectability (High)	The ability to detect small quantities of substance
EIA	Enzyme immunoassay
Polyclonal antibody preparation	An antibody preparation containing a number of antibodies to an antigen.
Sensitivity (High)	The ability to detect small changes in the amount of a substance
Titre	The concentration of antibody in a sample, expressed in terms of dilution

Table 5.2

Advantages and disadvantages of enzyme immunoassay

Advantages
Very high sensitivity, specificity and detectability are possible.
Detection equipment is relatively cheap.
No radiation hazards.
Reagents relatively cheap and of long shelf-life
Disadvantages
Many chromogens (particularly with horseradish peroxidase) are known carcinogens or mutagens. Some also have irritant properties.

5.1.2 Classification and designs of solid-phase enzyme immunoassays

EIA were developed in the mid-sixties for the localization of antigens in histological preparations (similar to immunofluorescence techniques) and for the identification of immunoelectrophoretic and immunodiffusion precipitation lines (Nakane & Pierce 1966, 1967; Avrameaus and Uriel, 1966). With the finding that immunoreactants could be adsorbed onto solid-phases, quantitative determination of antigens and antibodies become possible (Engvall & Perlmann, 1971; Van Weemen and Schuurs, 1971).

All solid-phase EIA procedures are based on the principles of Activity Amplification (AA) or Activity Modulation (AM). In AA assays, a large excess of immunoreactant is used to obtain a maximum signal for the test compound. The theoretical detection limit in these assays is one molecule, since even at low concentrations, most will react with an excess of immunoreactant by the law of mass action. In practice this has not been achieved and the detection limit is about 10^4 molecules. AM assays in contrast, depend upon competition of the test molecules for the same immunoreactant, to modulate the enzyme signal. The sensitivity of AM assays increases at lower immunoreactant concentrations since variations in the amount of test molecules effect a larger modulation of the enzyme signal. However at low antigen and antibody concentrations, complex formation is slow and accuracy is poor. In AM assays the immunoreactant is not in excess, and therefore reaction of antibody and antigen will be slower. Also detection limit is governed by the affinity constant of the antibody for the antigen. Calculations have shown that the detectability of an antibody/antigen complex with an affinity constant (Ka) of 10^{10} M^{-1} and a error of 5% was about 10^8 molecules/ml (Tijssen, 1985).

In conclusion AA assays are to be preterred, where possible, because of their greater sensitivity and speed.

5.1.2.1 Non-competitive solid-phase enzyme immunoassays

These assays are of the AA type and are amongst the most popular solid-phase EIA. They can be subdivided according to whether antigen or antibody is immobilized on the solid phase (for example, Fig 5.1 A,B). These two assays are called direct assays because the enzyme linked antibody combines directly with the antigen. Detectability may be increased by attaching the enzyme to an anti-IgG raised in another species. The antibody which binds to the antigen is not therefore labelled. This is called an indirect assay (Fig 5.1, C). Since one primary (unlabelled) antibody can interact with up to eight secondary (labelled) antibodies an amplified signal can be generated.

5.1.2.2 Competitive solid-phase enzyme immunoassays

The first solid-phase EIA described (Van Weeman & Schuurs, 1971; Engvall and Perlman, 1971) belonged to this group which is of the AM class. As with the non-competive solid-phase immunoassays, antigen or antibody can be immobilized. Where antigen is immobilized the antibody is labelled with enzyme. Standard antigen or test solution is added with labelled antibody and allowed to compete for the immobilized antigen (Fig 5.2, A). It is possible to increase the detectability of the above test by making it indirect. The test or standard antigen is preincubated with non-labelled antibody. A fraction of antibody binds antigen and is prevented from binding to immobilized antigen on the solid phase. The antibodies binding to the solid phase are detected by enzyme labelled anti-immunoglobulin antibodies or other methods (Fig 5.2, C) (5.1.3). This assay is therefore a mixed or sequential AM- and AA-

Figure 5.1

Non-competitive solid-phase EIA.

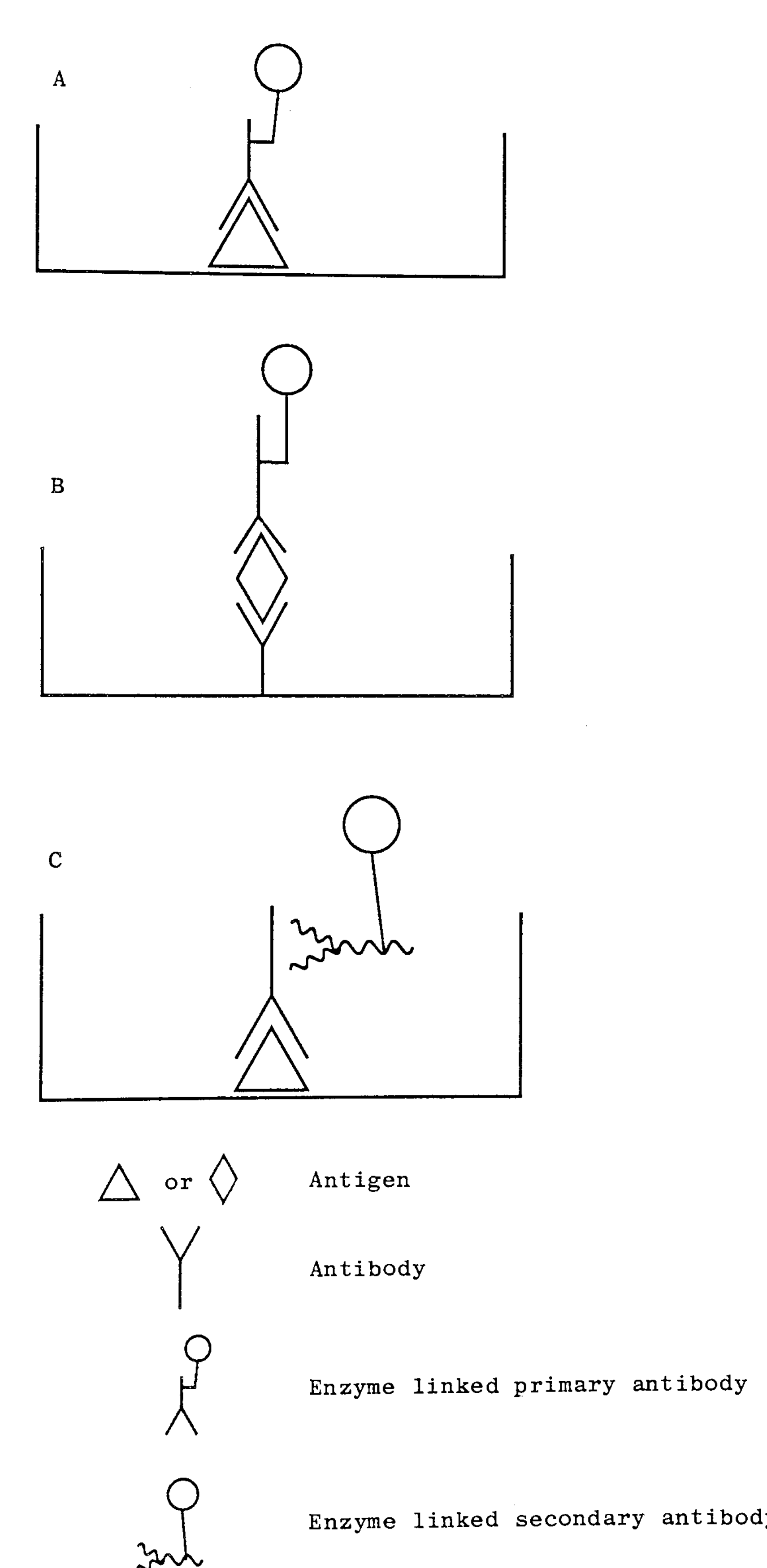
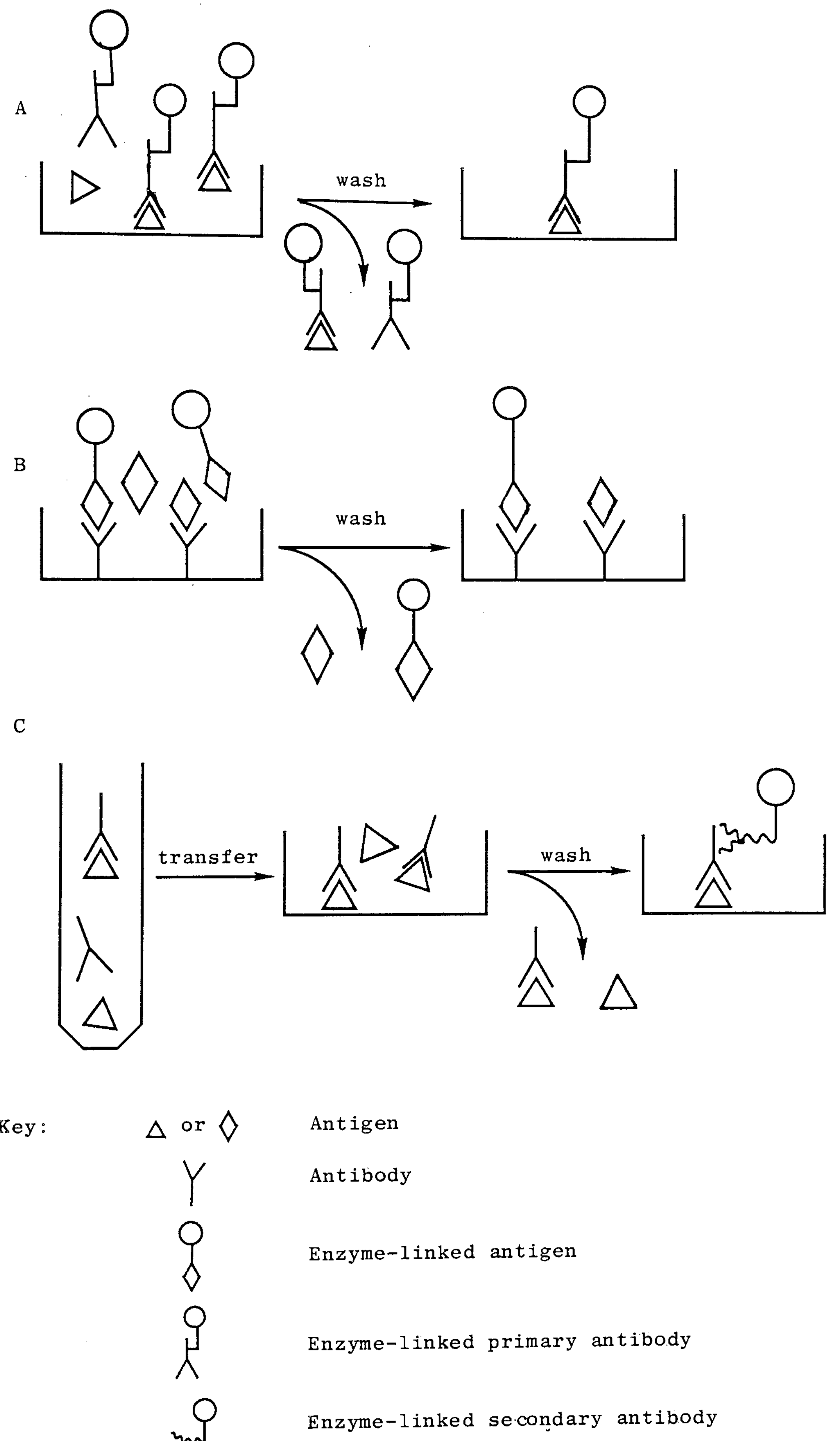


Figure 5.2

Competitive solid-phase EIA.



type procedure. (Tijssen and Kurstak, 1981). Where antibody is attached to the solid phase, antigen is labelled with enzyme. In this direct test, the binding of the antigen/enzyme conjugate is inhibited by the addition of labelled antigen. (Fig 5.2, B). The production of enzyme product is therefore inversely proportional to the concentration of free unlabelled antigen (Tijssen,1985).

5.1.3 Non-immunological recognition systems and enzyme labels used in immunoassays.

5.1.3.1 The avidin-biotin system

The use of a biotin-avidin-enzyme complex is rapidly becoming an important tool in solid-phase EIA since it produces superior detectabilities and low background levels. (+)-Biotin is one of several water soluble factors of the Vitamin B complex (Mr 244), and is a coenzyme for enzymes involved in carboxylation reactions. Avidin is a glycoprotein (67 kDa) comprised of four subunits (Green, 1975) and is isolated from egg white. Avidin has an exceptionally high affinity for (+)-biotin (Ka= $10^{15}M^{-1}$) and binds four molecules per molecule of avidin. (+) - biotin is very easily coupled to antibodies (biotinylation) and avidin can be linked to the various enzymes (via a spacing arm to prevent steric hindrance) used in solid phase EIA. Both of these modifications can be achieved without affecting antigen binding or enzymic activity. Since numerous biotin molecules can be attached to a single antibody, the biotinylated antibody will take up several enzyme-linked avidin molecules (essentially irreversibly) and thus amplify the signal produced by binding of antibody to the solid- phase. (Wilchek & Bayer, 1984; Shamsuddin & Harris, 1983). A protein very similar to avidin, streptavidin from <u>Streptomyces avidnii</u> is now preferred to avidin. It has the same biotin binding properties as avidin, but is reported to cause lower background staining because it lacks the carbohydrate moiety present on avidin.

5.1.3.2 Protein A

Protein A, isolated from the cell wall of <u>Staphylococcus aureus</u> (usually Cowan 1 strain) binds strongly (K_a rabbit immunoglobulin G 10^8M^{-1}; Langone, 1982) to immunoglobulin G molecules at the interface between the C_H2-C_H3 regions in human and guinea pig (Langone, 1982). Consequently the immunoglobulin/protein A complex does not inhibit antibody/antigen interaction. Protein A is usually conjugated to an enzyme label. The protein contains four highly homologous domains each capable of binding immunoglobulin (Sjodall, 1977) though only two sites usually react.

5.1.3.3 Enzyme labels commonly used in solid-phase enzyme immunoassays.

No one enzyme fulfills all the criteria for an ideal label in EIA (Table 5.3) and a compromise is usually made. The two most popular enzyme labels are alkaline phosphatase (APase) and in particular horse radish-peroxidase (POase). Both enzymes are cheap, have high specific activity, are stably conjugated to other proteins and are easily obtainable commercially (Tijssen, 1985).

POase (hydrogen-peroxide oxidoreductase, EC 1.11.1.7) is the most widely used enzyme label in EIA. Typical POases are haemoproteins and transfer hydrogen from hydrogen donors to hydrogen peroxide. Almost without exception, POase activity is measured indirectly by the rate of transformation of the hydrogen donor (a chromogen for colourimetric assays) (Tijssen, 1985).

An example of a chromogen which is used to determine POase activity is O-phenylenediamine (OPD) which when oxidised forms an orange compound with

Table 5.3

Properties which make an enzyme ideal for EIA

High turnover number
Low K_M for substrate, but high K_M for product
Stable upon storage (in free or conjugated form)
Easily detectable activity
Absence of endogenous enzyme or interfering substances in the sample

absorption maxima at 445nm and 492nm. OPD is mutagenic and the enzyme is inhibited by sodium azide (Tijssen, 1985).

The APases (orthophosphoric monoester phosphohydrolase, alkaline optimum, EC 3.1.3.1) used in EIA procedures are isolated from bovine intestinal mucosa or from E. coli (Tijssen, 1985). In colourimetric determinations of APase activity the use of p-nitrophenyl phosphate (p-NPP) as a substrate is almost universal. The choice of this compound is popular since; (i) its spontaneous hydrolysis is low below 30^0C; and (ii) its hydrolysis product p-nitrophenol absorbs strongly at 405nm. Although p-nitrophenol is not mutagenic, the molar extinction coefficient is lower than that of POase substrates such as OPD (Ishikawa et al, 1983).

5.1.4 Immunoassays for gastrointestinal mucin

Forstner et al., (1977) measured rat small intestinal goblet cell mucin by radioimmunoassay. Antibodies to rat goblet cell mucin were raised in rabbits and standard mucin was isotopically labelled with [^{3}H]. Tracer amounts of labelled mucin were then incubated with the anti-rat goblet cell mucin antibody preparation in the presence of unlabelled mucin standards or sample. The resulting complexes were precipitated with sheep anti-rabbit immunoglobulin G antiserum. Some features of this assay are presented in Table 5.4.

A more convenient solid-phase radioimmunoassay for goblet cell mucin was subsequently developed Roomi et al., (1984). In this procedure the wells of a microtitre plate were coated with a fixed concentration of mucin. To this a portion of preincubation solution (antibody incubated with standard mucin or test sample) was added. Antibody binding to the plate was therefore inversely proportional to the mucin in the preincubation solution and was detected using [^{125}I]-

Table 5.4

Immunoassays for gastrointestinal mucin

Author	Assay type	Range ng mucin/ sample	Coefficient of variation Intraassay	Interassay
Forstner, (1977)	Double antibody RIA	3-80	-	-
Roomi et al., (1984)	Solid-phase RIA	6-100	~5%	~10%
Mantle & Thakore, (1988)	Solid-phase EIA (indirect competitive)	8-250	-	8%
Roumagnac & Laboisse, (1989)	Solid-Phase EIA (indirect non-competitive)	~2-16	-	10%
McCool et al., (1990)	Solid-phase EIA (indirect (non-competitive)	~20-2000	-	-

labelled protein A. By comparison with the previous solution assay this solid-phase assay required no isotopic labelling of either the antigen or antibody, used less antibody and could perform a large number of determinations simultaneously. Some parameters of this method are shown in Table 5.4. An identical protocol to this was used by Mantle et al., (1984) to investigate the antigenic and structural features of human intestinal goblet cell mucin.

The measurement of mucin secretion and tissue mucin content in rabbit small intestine and colon by an indirect competitive solid-phase EIA was reported by Mantle & Thakore, (1988). This technique was based upon the earlier solid-phase radioimmunoassay (Roomi et al, 1984) except that protein A was conjugated to horse-radish peroxidase rather than [^{125}I]-labelled (c.f. Fig 5.2 C). The assay was very sensitive (Table 5.4). Since this procedure required no isotopically labelled components, it represents a considerable advance on the solid phase radioimmunoassay.

More recently, a simple immunofiltration assay for the measurement of human colonic mucin, secreted by the HT-29 epithelial cell line has been reported by Roumagnac and Laboisse, (1989). In this procedure, standard mucin or test sample were immobilized under vacuum on a specially designed microtitre plate, in which the bottom of each well was formed by a sheet of nitrocellulose. Each well was then incubated with primary antibody solution and unbound antibody was removed by aspiration. Each well was then incubated with secondary antibody conjugated with horse-radish peroxidase (c.f. Fig 5.1 C). Bound enzyme activity was assessed using O-phenylenediamine as substrate. After colour development a portion of reaction mixture from each well was transferred to a microtitre plate and the absorbance at 490nm was read. Some features of the assay are given in Table 5.4. Although the amount of mucin detectable with this assay was not as good as the

indirect competitive EIA (Mantle & Thakore, 1988) it does illustrate the numerous potential protocols that are available with enzyme immunoassay methodology.

A novel solid-phase EIA has been recently reported by McCool et al., (1990) for measuring human small intestinal mucin secreted from T84 human colonic adenocarcinoma cells. Mucin containing samples were adsorbed onto a nitrocellulose sheet which was incubated with anti-mucin antibody. Excess antibody was removed by washing and the sheet was then incubated with protein A - horse-radish peroxidase conjugate (c.f. Fig 5.1 C). Bound enzyme activity was revealed using hydrogen peroxide/4-chloro-1-naphthol as substrate and colour development was assessed using a densitometer. Some characteristics of this assay are given in Table 5.4.

Comparison of the various assays (Table 5.4) suggests that for sensitivity, range of measurement and ease of performance the indirect competitive solid-phase EIA is to be preferred.

5.1.5 Choice of solid-phase EIA suitable for quantitating rat gastric mucin secretion

One of the aims of this section of work was to develop a suitable solid-phase enzyme immunoassay to quantitate secreted rat gastric mucin. An indirect competitive solid-phase assay in which antibody bound to immobilized antigen was detected with protein A-alkaline phosphatase conjugate was chosen for the following reasons: Firstly, it was uncertain whether secreted mucin could be quantitatively bound to a solid phase and it was desirable to avoid enzyme labelling of the antibody preparation since this would introduce added experimental complications. Thus, a competitive assay was chosen. Secondly, the assay was made indirect as enzyme labelling of the antibody preparation would be obviated and the use of an enzyme labelled secondary antibody should improve

detectability. Finally, alkaline phosphatase was preferred to horse-radish peroxidase as enzyme because sodium azide which would be present in samples inhibits the latter enzyme. Furthermore, alkaline phosphatase conjugated to protein A was preferred to enzyme labelled anti-rabbit IgG since an indirect competitive procedure to measure small intestinal mucin with [^{125}I]-labelled protein A (Roomi et al., 1984) was very successful.

5.1.6 Assessment of antibody specificity

The specificity of an antiserum preparation is of vital importance when it is used in quantitative immunological assay procedures. In this study three methods have been employed to determine immunological specifity: (i) Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) coupled with protein blotting; (ii) Immunohistochemical techniques (including immunofluorescence and immunoperoxidase labelling); and (iii) analysis by indirect competitive solid-phase enzyme immunoassay. The advantages of each system are outlined below.

5.1.6.1 SDS-PAGE and protein blotting

Proteins are separated by electrophoresis on polyacrylamide gels in the presence of the detergent sodium dodecyl sulphate and are transferred to a nitrocellulose membrane (protein or Western blotting; Towbin et al, 1979). The protein blot, unlike the gel, can be probed with antiserum preparation and binding sites localized. Since SDS-PAGE separates proteins according to their relative molecular mass, antibodies that are highly specific for one antigenic site (epitope) should show one discrete band. Multiple staining bands indicates either that the antibody preparation recognises more than one epitope or that a single epitope appears on more than one protein. Procedures for the detection of antibody binding are similar to those discussed previously (5.1.3).

5.1.6.2 Immunohistochemistry

Antibodies are conjugated with a fluorochrome usually fluorescein isothiocyanate (FITC) which gives an emission at 525nm (green) (Pearse, 1980) Fluorescein is a popular choice for this technique since it is stable and its emissions are bright, however, its fluorescence fades rapidly. Addition of an anti-fading compound to the mounting medium such as 1,4 diazabicyclo-(2,2,2)-octane (DABCO; Johnson & Holborow) which is stable and non-ionizing can overcome this problem.

Two procedures are possible for immunofluorescence analysis of tissue preparations such as whole cells or sections: (i) the direct method; and (ii) the indirect method.

In the direct method, the primary antibody (ie that which recognizes the antigen) is directly conjugated to FITC. In the indirect method, a secondary antibody (i.e. an antibody capable of recognizing the Fc portion of the primary antibody) is conjugated to FITC. As mentioned previously (5.1.2.1) indirect methods are more sensitive.

However, the indirect method can increase background fluorescence due to non-specific binding of the secondary antibody. In this investigation indirect immunofluorescence was used to establish antiserum specificity.

Anti-immunoglobulin G can be conjugated with enzymes such as horse-radish peroxidase (Avrameas & Uriel, 1966; Nakane & Pierce, 1966) rather than to a fluorochrome. The relative advantages of the two procedures are compared in Table 5.5. An amplified signal can be achieved by the use of the biotin-avidin link (5.1.3.1).

Table 5.5

Advantages and disadvantages of immunoperoxidase labelling verses immunofluorescence labelling

Advantages
Normal light microscope can be used
Can be used at ultrastructural level
Detectability in some procedures is significnatly higher (>100X)
Less background staining
Permanent record
Long shelf life of reagents
Disadvantages
May be more expensive
Some compounds may be mutagenic
Assay is more complicated

The major advantage of these procedures is that the cell-type associated with the antigen can be distinguished. Potential problems, however, are the modification of epitopes during fixation and access of the antibody preparation to some antigenic sites, for example, inside whole cells.

5.1.6.3 Indirect competitive solid-phase EIA

The final method used in this investigation to determine antiserum specificity was an indirect competitive solid-phase enzyme immunoassay. This procedure can be used to assess the tissue and species distribution of epitopes recognized by the antibody preparation. A narrow distribution (see Mantle & Thakore, 1988) would be consistent with a specific preparation.

5.1.7 Aims of this section

At the start of this study (1987), the development of a solid-phase EIA to accurately quantitate secreted rat gastric mucin had not been achieved, and since then although a procedure has been published for small intestinal mucin (Mantle & Thakore, 1988) no other procedure for gastric mucin has been detailed. This work was therefore undertaken with the following aims:

1) The production of a specific antiserum to 'native' rat gastric mucin in Netherland Dwarf rabbits.

2) The development of a suitable solid-phase EIA to quantitate secreted gastric mucin that would enable more samples to be assayed simultaneously than was possible with the [^{3}H]-labelling methodology and without prior purification.

5.2 METHODOLOGY

5.2.1 Polyclonal antiserum production

5.2.1.1 Preparation of adjuvant/immunogen mixture

Rat gastric mucin was purified as described previously (see 2.3). Freeze-dried mucin was resuspended to twice the required concentration for innoculation in phosphate buffered saline (composition: 150mM-sodium chloride, 20mM-sodium phosphate, pH7.0). To this solution an equal volume of adjuvant (Freund's complete or incomplete see 5.2.1.2) was added. The adjuvant/mucin mixture was sonicated for six 5 second periods at 20W with a Soniprep 150 (MSE, Croydon) to form a stable emulsion.

5.2.1.2 Immunization protocol

A sample of blood was removed from a Netherland Dwarf rabbit to provide preimmune control serum (5.2.1.3), then the rabbit was given a primary innoculation containing 50µg of rat gastric mucin in Freund's complete adjuvant (1ml) by subcutaneous injection at three sites on the back. Subsequently 50µg of mucin in Freund's incomplete adjuvant (1ml) was administered similarly every two weeks for eight weeks. After this period the rabbit was test-bled (5.2.1.3) and analysed for antibodies to rat gastric mucin by Ouchterlony double diffusion (5.2.2). A further immunization period was instigated under the same procedure using 200µg of mucin for another twelve weeks. After this period an antibody fraction was isolated and purified as described in 5.2.1.4.

5.2.1.3 Bleeding procedures

The blood required for preimmune serum and test-bleed serum (approximately 5ml) was obtained by bleeding from the marginal ear vein. The posterior side and the edge of one of the ears was shaved, dried

and smeared with a thin layer of petroleum jelly. A diagonal cut was made across the vein (not severing it) with a scalpel blade and a plastic test tube was placed immediately under the ear for collection of blood.

Blood for preparation of the antibody fraction (5.2.1.4) was obtained by vacuum exsanguination from the heart under terminal anaesthesia.

Blood was allowed to clot for 2h at room temperature and then left overnight at 4^0C. The serum was decanted and clarified by centrifugation at $15,000g_{AV}$ for 15min. Clarified serum was then stored at -70^0C.

5.2.1.4 Preparation of an immunoglobulin fraction from serum

An immunoglobulin fraction was prepared from serum by batch treatment with DE52 cellulose anion exchanger (Whatman, Maidstone) at pH7.5, followed by precipitation with ammonium sulphate at 45% saturation.

25g of pre-swollen DE52 cellulose was equilibrated by adding 150ml of 200mM-sodium phosphate buffer (pH 7.5) for 5 min at 4^0C. The pH of the resulting buffer/ion exchange slurry was adjusted to pH 7.5 with the addition of sodium dihydrogen orthophosphate whilst stirring. The slurry was allowed to settle and the supernatant with fines were decanted off. The ion exchanger was redispersed with 10mM-sodium phosphate buffer pH 7.5 to a total volume of 150ml. The ion exchanger was allowed to settle and the supernatant was decanted off. This process was repeated once more. Finally the ion exchanger was redispersed into 150ml 10mM-phosphate buffer and filtered (Buchner funnel with three Whatman No. 1 filter papers) using a vaccum pump, and washed with 100ml of 10mM-sodium phosphate buffer.

Serum (32ml) was added to the equilibrated wet ion exchanger in the ratio of 1ml serum to 1.33g wet DE52 cellulose and left at 4^0C for 30 min with occasional stirring. After equilibration the serum/DE52 cellulose mixture was filtered as above and washed with 10mM-sodium phosphate buffer equivalent to the volume of the serum processed. The filtrate and wash were retained.

Solid ammonium sulphate was gradually added to the pooled filtrate and wash, with continuous stirring to 45% saturation (0.262g/ml solution) at 4^0C. The solution was then stirred for 30min at 4^0C, followed by centrifugation at $15,000g_{AV}$ for 15min. The resulting pellet was resuspended in 4.75ml of 0.15M-sodium chloride, 20mM-sodium phosphate at pH 7.0 to give a concentrated, partially purified, antibody fraction. This was dialysed versus two changes of 21 of the same buffer to remove remaining ammonium sulphate, and stored as aliquots at -70^0C.

5.2.2 Ouchterlony double-diffusion

1% (w/v) agarose stock solutions (16ml) were made up in 0.15M-sodium chloride, 20mM-sodium phosphate pH 7.0 at 100^0C in a boiling water bath. These were allowed to set at room temperature and stored at 4^0C.

When required stock agarose solutions were melted in a boiling water bath and transferred to water bath at 60^0C. A clean glass plate (101mm x 82mm) was levelled on a levelling table (LKB, Milton Keynes). Melted agarose solution was poured onto the plate, distributed evenly over its surface, and left to set at room temperature.

The required pattern of wells was cut into the set gel with a 4mm gel punch (L K B, Milton Keynes) attached to a vacuum line and 20µl of serum from test bleed or purified rat gastric mucin solutions were added. The plate was incubated at room temperature for 24h in a humid chamber.

After incubation the gel was removed from the glass plate and washed in saline overnight. After washing the gel was placed on the hydrophillic side of a Gelbond film (Sigma, Poole) and pressed under ten sheets of filter paper (No 1, Whatman, Maidstone).

After pressing the gel was dried onto a film with a hairdryer and stained with 0.25% (w/v) Page Blue 83 in 45% (v/v) methanol, 9% (v/v) acetic acid solution for 10min with shaking. After staining the gel was destained in 20% (v/v) methanol, 5% (v/v) acetic acid solution until the background was colourless. Finally the gel was dried with a hairdryer.

5.2.3 Protein blotting

Isolated rat stomach cells were incubated for 60 min at 37^0C in the presence of 50µM-forskolin and with the bovine serum albumin concentration in the medium reduced to 0.1mg/ml. Cell suspension (2ml) was centrifuged at 12,000g for 30 s and the supernatant dialysed against distilled water for 18 h at 4^0C. A portion (1.5ml) of the dialysate was freeze-dried (Modulyo, Edwards, Crawley,) and stored at -20^0C.

When required, secreted material or purified rat gastric mucin were heated for 5 min at 100^0C in electrophoresis sample buffer, without reduction of disulphide bridges. Samples were separated by non-reducing SDS-PAGE as described in 2.5.

5.2.3.1 Electrophoretic transfer to nitrocellulose membranes

The Novoblot horizontal electrophoretic transfer unit (LKB, Milton Keynes) was prepared for the transfer procedure by soaking both graphite electrodes with distilled water. Nine filter papers (Grade 1F, LKB, Milton Keynes) cut to the same size as the gel were soaked with continuous transfer buffer (composition: 39mM-glycine, 48mM-Tris, 1.3mM-SDS and 20% (v/v)

methanol pH 7.6). These were placed on the anode avoiding trapping any air bubbles. A nitrocellulose membrane (Hybond C, Amersham, International, Amersham) was soaked with transfer buffer. After SDS-PAGE one of the glass plates holding the gel was removed, and the wet nitrocellulose membrane was placed on top of the whole gel. The glass plate/gel/nitrocellulose sandwich was inverted and the glass plate was removed avoiding trapping air between the nitrocellulose and the gel. The gel/nitrocellulose unit was placed on the blotting papers on the anode (with the gel uppermost). A further nine filter papers were soaked with transfer buffer and added to the top of the gel avoiding trapping any air bubbles. The cathode was then placed on top of the upper filter papers.

The transfer unit was operated at a constant current calculated as follows:

$$\text{Electrophoretic transfer current (mA)} = 0.8 \times \text{surface area of gel (cm}^2\text{)}$$

After 1h the cathode plate was carefully removed and the filter paper/nitrocellulose membrane/gel stack was resoaked with transfer buffer. Electrophoretic transfer was then continued for another 1h.

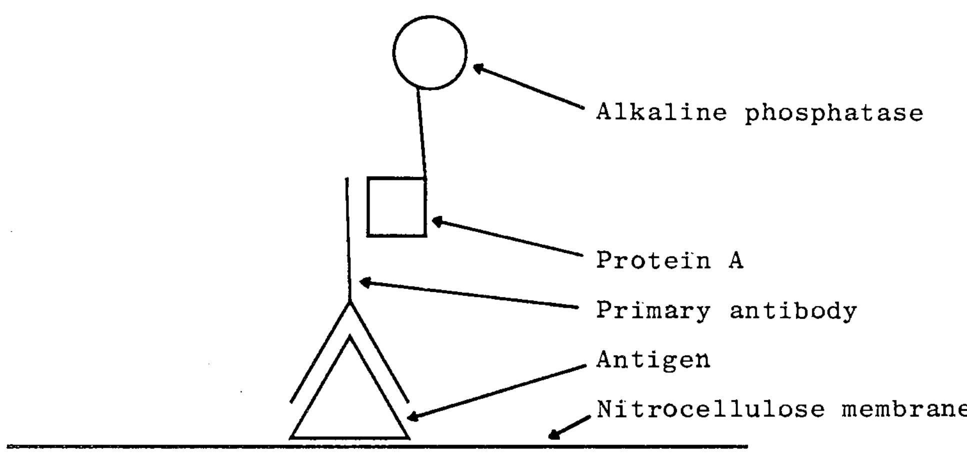
Finally the nitrocellulose sheet was carefully removed, air dried and stored at 4^0C in a 250ml polythene container in the presence of dessicant.

5.2.3.2 Immunodetection (Fig 5.3)

The membrane was rehydrated with 10ml of Tris buffered saline (TBS; 146mM-sodium chloride, 20mM-Tris, pH 7.6) in a Sterilin plastic container at room temperature on a rock and roll table (Luckham, Burgess Hill) for 10 min at 60 r.p.m.

Figure 5.3

Schematic representation of protein blotting using protein A-alkaline phosphatase conjugate detection.



Non-specific binding sites on the membrane were then blocked with 10ml 5% (w/v) de-fatted dried milk powder (Marvel, Cadbury) in TBS-0.5% (v/v) Tween 20; pH 7.6. Incubation was for 2h at room temperature using the rock and roll table at 60 r.p.m. The membrane was washed for 1 x 1 min then 3 x 15 min with 20ml TBS-0.1% (v/v) Tween 20 at 100 r.p.m. All subsequent washing steps were carried out similarly.

Following the wash, 10ml anti-rat gastric mucin antibody preparation or preimmune serum (1:1000 dilution) in TBS-0.1% (v/v) Tween 20, 1% (w/v) dried milk powder was added to the container. Incubation was for 1h at room temperature at 60 r.p.m.

Subsequent to another wash cycle, 10ml TBS-0.1%(v/v) Tween 20, 1% (w/v) dried milk powder containing 5µg protein A-alkaline phosphatase conjugate was added. Incubation conditions were the same as that for the primary antibody.

After a further wash cycle, the membrane was removed from the polystyrene container and placed in a 15 x 18.5 x 3 cm polythene tray. Substrate solution (50ml) containing 116mM-Nitro blue tetrazolium, 110mM-5-Bromo-4-chloro-3-indolyl phosphate in diethanolamine buffer (composition: 100mM-diethanolamine, 10mM-magnesium chloride pH 9.5) was added to the tray. Incubation was for 20min at room temperature with gentle shaking. After colour development the membrane was washed with double-distilled water for 3 x 10min at room temperature with shaking. The developed membranes were air-dried and stored at room temperature.

5.2.4 Immunohistochemistry (performed in collaboration with J.M. Williams)

5.2.4.1 Preparation of smears

Isolated cells (prepared as described in 2.1) were resuspended with medium consisting of Eagles minimum essential medium plus 20mM-HEPES and 1mg/ml bovine serum albumin (pH 7.35) at a concentration of 5 x 10^7 cells/ml. A droplet (10μl) of cell suspension was placed on a clean microscope slide and smeared alaong its length. The slide was allowed to air-dry before fixation in acetone at room temperature for 1min. The slide was allowed to dry in air for 1min before storage at -20^0C in air-tight bags.

5.2.4.2 Tissue preparation and paraffin-embedding

A Wistar rat (starved overnight) was anaesthetised with an interperitoneal injection of sodium pentobarbitone (60mg/kg). A midline incision was made to expose the stomach. The gastrointestinal tract was ligated proximal and distal to the stomach which was then excised and washed in ice-cold saline (0.9g/l). Injection of saline between the muscle and mucosa of the fundic area of the organ induced a blister from which the muscle layer was cut away. The underlying mucosa was removed and placed on nitrocellulose discs which were placed in plastic histological cases to prevent folding of the tissue.

Tissue was initially fixed in 95% ethanol at 4^0C for 1h. Following this the specimen underwent 3 changes of absolute alcohol at 4^0C for 2h and a single change overnight at 4^0C. After this two changes of xylene at 4^0C for 1h were administered followed by one change for 1h at room temperature. Finally the sample underwent paraffin immersion for 1h at 56^0C four times and the resulting blocks were stored at 4^0C until required.

Blocks were sectioned with a microtome (Spencer 820, American Optical Co., U.S.A.) at 5µm. After brief floatation on water at 40^{0}C the sections were dried in an incubator at 37^{0}C for at least 30min. Slices were deparaffinised by two immersions in xylene for 1min followed by three changes in cold 95% ethanol for 1min each to achieve clearing. The sections were finally washed in cold phosphate-buffered saline (composition: 0.14M-sodium chloride, 2.7mM-potassium chloride, 1.5mM-potassium dihydrogen orthophosphate, 8.1mM-disodium hydrogen orthophosphate, pH 7.4) for 1min three times. Sections were subsequently stored at room temperature.

5.2.4.3 Immunofluorescence of isolated cell smears

Frozen slides were brought to room temperature in their air-tight bags and then allowed to dry. A test area was established on the slide by painting a ring of approximately 1cm diameter with Tipp-Ex. The anti-rat gastric mucin antibody preparation was diluted 1/5 with phosphate buffered saline (PBS) containing 1% (w/v) bovine serum albumin (BSA) and 100µl was applied and left for 30min at room temperature in a humid chamber. The primary antibody preparation was rinsed off with PBS containing 1% (w/v) BSA from a wash bottle and then washed three times for 10min each by immersion in the same solution. After washing the sides of the slide were blotted dry but the test area was kept moist. Secondary antibody:- FITC conjugated sheep anti-rabbit immunoglobulin G (100µl) diluted 1/10 with 1% (w/v) BSA in PBS, was then applied for 30min at room temperature in a humid chamber. After incubation the slide was washed as described above.

The slide was mounted in 10µl glycerol containing 10% (v/v) PBS and 2.5% (w/v) DABCO (pH 8.9) under a coverslip and viewed under a fluorescent microscope (Jenamed) fitted with 35mm single lens reflex camera (OM10, Olympus). Incident radiation was over the range 450-490nm and fluorescence was monitored at 525nm.

5.2.4.4 Immunoperoxidase labelling of paraffin-embedded tissue sections (Fig 5.4)

Paraffin sections were immersed for 10-15min in a solution of 0.5% hydrogen peroxide in methanol (freshly prepared) to destroy endogenous peroxidase enzyme activity. The sections were washed twice in PBS and once in 0.2% (w/v) gelatin in PBS each for 5min. The primary antibody preparation (or preimmune control serum) was applied to the specimen at a dilution of 1/500 and incubated at room temperature for 30min in a humid chamber. After incubation the sample was washed three times with 0.2% (w/v) gelatin in PBS for 10min each. The secondary antibody (biotinylated donkey anti-rabbit immunoglobulin G; Amersham International, Amersham) was added at a dilution of 1/50 again for 30 min at room temperature in a humid chamber. After washing as before, ExtrAvidin (avidin-peroxidase conjugate, Sigma, Poole) was applied for 15min at a dilution of 1/150. Specimens were washed as previously and the freshly-made substrate solution (0.06% (w/v) diaminobenzidine, and 0.01% (v/v) hydrogen peroxide in PBS containing 1% (w/v) BSA) was added for 10min. The reaction was stopped by washing under tap water for a few minutes.

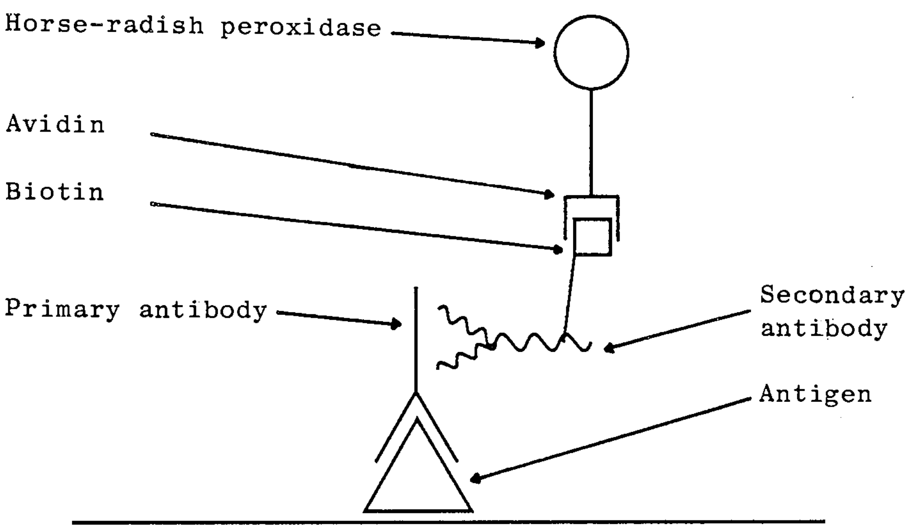
Sections then underwent three changes of absolute alcohol for 1min each, followed by three changes of xylene for 1min each and were mounted in DPX (BDH, Poole) under a coverslip. These were then viewed under a light microscope.

5.2.5 'Checker-board' ELISA protocol

The procedure used for the 'checker-board' ELISA is similar to that described by Tijssen, (1985). Purified rat gastric mucin was serially diluted by placing 50µl of PBS in each well of an ELISA microtitre plate (Dynatech, U.S.A). To each well in column 2 50µl of PBS containing 12.8µg mucin was added. The solution was thoroughly mixed by pipette (Gilson, Luton). A

Figure 5.4

Schematic representation of the indirect immunoperoxidase labelling technique.



For the sake of clarity only one biotin molecule is shown attached to the secondary antibody in the diagram above. It should be emphasized that several biotin molecules can be conjugated to one secondary antibody thus providing an amplified signal.

portion (50µl) of each solution was transferred to the wells in column 3 and mixed as before. This process was continued until column 12 was reached where after mixing 50µl of solution was discarded. The plate was wrapped in plastic film (Clingfilm) and incubated overnight at 4^{0}C. The plate was then emptied by shaking it over a sink and banging the plate on a wad of absorbent paper three times. The plate was then flooded with PBS containing 0.1% (v/v) Tween 20 from a wash bottle and allowed to stand for 3min. This procedure was repeated twice.

Remaining non-specific binding sites on the plate were blocked by incubation with 4mg BSA in 50µl PBS. The plate was covered with clingfilm and left for 1h at room temperature. After the incubation period the plate was washed as previously described.

After washing, 50µl of serially diluted antibody preparation (1:2 dilutions in PBS, starting dilution 1:100) was added to each horizontal row starting with row B, i.e. row B contained 1:100 diluted antibody preparation, row C 1:200 etc. The plate was then incubated for 1h at room temperature covered with Clingfilm. After incubation the plate was washed as described earlier.

Subsequent to washing, 50µl Tris-buffered saline (composition as for PBS except that 20mM-Tris replaced phosphates), containing 50ng protein A-alkaline phosphatase conjugate was added to each well. The plate was wrapped in Clingfilm and left for 1h at room temperature. After incubation the plate was washed with Tris-buffered saline containing 0.1% (v/v) Tween 20 as before.

Finally 150µg of disodium p-nitrophenyl phosphate in 150µl diethanolamine buffer (composition: 1M-diethanolamine, 0.5mM-magnesium chloride, pH 9.8) was added to each well. Colour development was stopped after 1h by the addition of 3M-sodium hydroxide (50µl).

Absorbance in each well was determined at 405nm using an ELISA plate reader (Anthos 2001, Anthos Labtec Instruments, Austria).

5.2.6 Indirect competitive solid-phase EIA protocol

The procedure used for the indirect competitive solid-phase EIA is outlined in the Flow diagram (Fig. 5.5). A solution containing 4mg BSA in 200µl of PBS was added to the wells of a round-bottomed microtitre plate (Flow Laboratories, Rickmansworth). The plate was covered with Clingfilm and left overnight at 4^0C to block non-specific binding sites. This preincubation plate was washed after incubation as described in 5.2.5 with PBS containing 0.1% (v/v) Tween 20. A portion (40µl) of antibody preparation at its working dilution and containing 1µg/ml pepstatin A, 1µg/ml leupeptin, 5mM-EDTA, 0.1mM-phenylmethylsulphonyl fluoride and 0.02% (w/v) sodium azide was added to each well except the blank wells (Fig. 5.6). To this an equivalent portion of sample or purified mucin standard was then added to each well except the blank and zero sample wells. The preincubation plate was then incubated either at room temperature for 2h or 37^0C overnight in a humid chamber.

The wells of an ELISA microtitre plate (Dynatech U.S.A) were coated with 400ng of purified mucin in 50µl PBS and incubated overnight at 4^0C. This plate was washed and then treated with 4mg BSA in 200µl of PBS for 1h at room temperature. The plate was washed and 50µl of the antibody/antigen mixture was transferred from the preincubation plate to the ELISA plate, and left for 1h at room temperature. After washing, 50ng of protein A-alkaline phosphatase conjugate in 50µl of Tris-buffered saline was added to each well. Subsequent steps were as described in 5.2.5.

Figure 5.5

A schematic representation of the final assay protocol for 'native' rat gastric mucin.

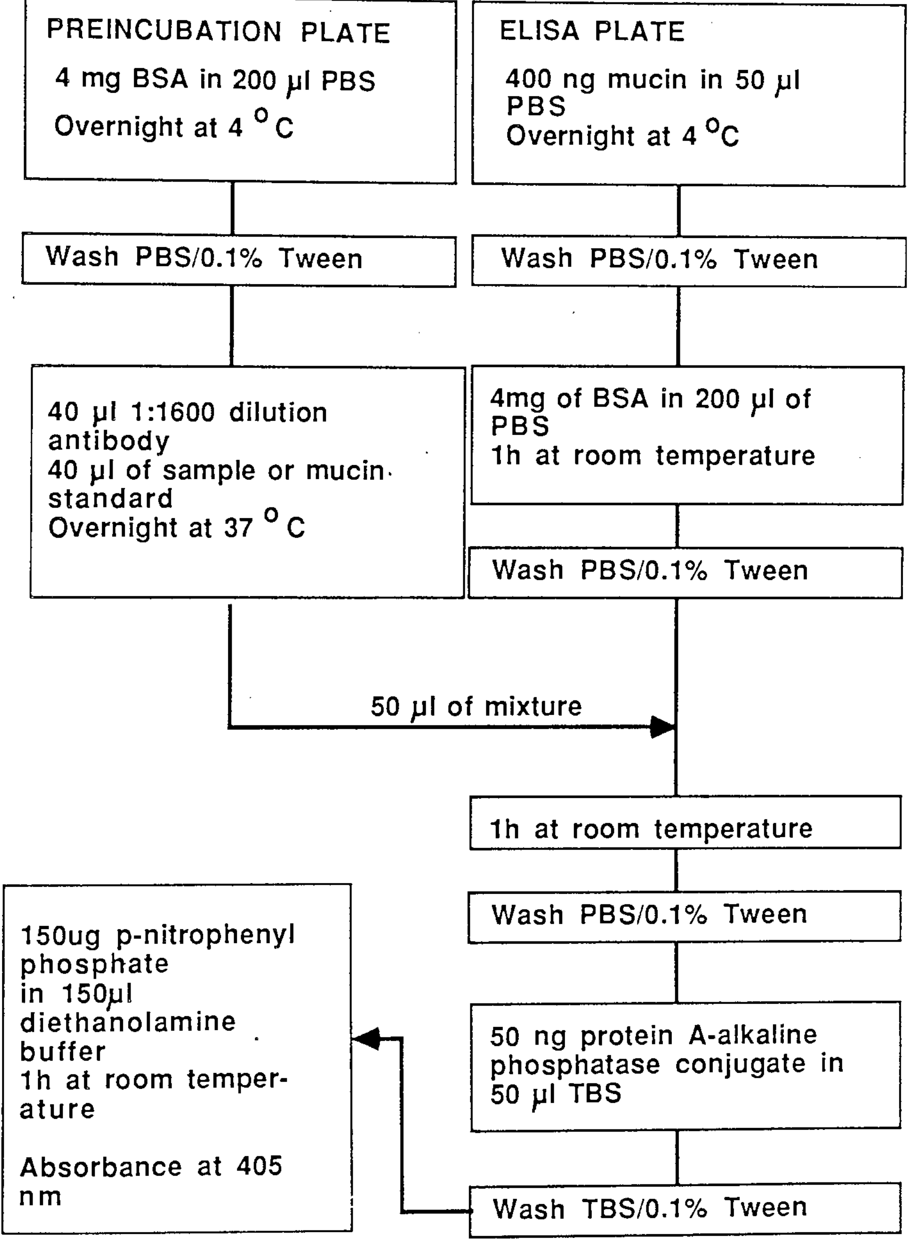


Figure 5.6

Disposition of samples on preincubation/ELISA plate

	1	2	3	4	5	6	7	8	9	10	11	12
A	B	S7	01	02	03	B	S7	01	02	03	BS7	
B	Ab					Ab					Ab	
C	S1					S1					S1	
D	S2					S2					S2	
E	S3					S3					S3	
F	S4					S4					S4	
G	S5					S5					S5	
H	S6					S6					S6	

Where
B: Blank
Ab: Antibody without antigen (zero sample)
S1-S7: Standards with mucin concentrations of 1.6, 4.8, 14.4 ...... 1166.4ng/40µl
01-03: Experimental samples taken at t=0

The samples of experimental material from each incubation vial (three vials for the controls and two vials for treatments) were distributed in the remaining wells.

5.2.7 Antiserum to reduced-denatured rat gastric mucin

A rabbit polyclonal antiserum to 'reduced-denatured' rat gastric mucin was kindly provided by Dr Ger J. Strous.

5.3 RESULTS AND DISCUSSION

5.3.1 Rabbit anti-rat gastric mucin polyclonal antiserum production and specificity

5.3.1.1 Assessment of mucin purity

Partially purified preparations of 'native' rat gastric mucin and reduced, carboxymethylated and denatured mucin did not contain any contaminating protein material of molecular mass less than approximately 200 kDa when examined by SDS-PAGE followed by silver staining of the gel (Plate 5.1; see 2.5 for methodology). A band running at approximately 57 kDa was present in all lanes on the gel (including those containing sample buffer only) and is therefore likely to represent an artefact.

5.3.1.2 Immunization protocol

Analysis of rabbit serum obtained after eight weeks of immunization by using Ouchterlony double-diffusion exhibited a very faint cross-reaction to partially purified rat gastric mucin. Immunization was therefore continued using more antigen as described in the methods (5.2.1.2). After a further twelve weeks the presence of a high titre of antibodies to rat gastric mucin was picked up by 'checker board' ELISA (5.3.2.1). The specificity of the antibody preparation was then investigated.

5.3.1.3 Protein blotting

The partially purified rabbit polyclonal antibody preparation was found to recognize rat gastric mucin on blots of polyacrylamide gels (Fig 5.7). A single sharp band was present at the top of the stacking gel, a diffuse band of staining material was present in the stacking gel and staining was also evident at the stacking gel / separating gel interface. Antibody preparations against rat gastric mucin (Dekker et al,

Plate 5.1

SDS-PAGE and silver staining of purified 'native' rat gastric mucin, reduced carboxymethylated and denatued rat gastric mucin and incubation medium from a suspension of cells stimulated with 50μM-forskolin.

Results of SDS-PAGE of 2μg and 270ng of 'native' rat gastric mucin (lanes 8 and 7 respectively), of 2μg and 270ng of ' reduced-denatured' rat gastric mucin (lanes 6 and 5 respectively) and of incubation medium (lane 4). Lanes 1-3 are blank controls and lanes 9 and 10 contain Sigma SDS-PAGE marker proteins (1:6 diluted) and Amersham Rainbow molecular weight markers respectively. The arrow represents the division between the stacking gel (4% acrylamide) and the separating gel (8% acrylamide). The numbers and associated lines represent the molecular mass (kDa) and the positions of the Rainbow marker proteins myosin, phosphorylase b, bovine serum albumin, ovalbumin and carbonic anhydrase (which ran with the solvent front).

Plate 5.1

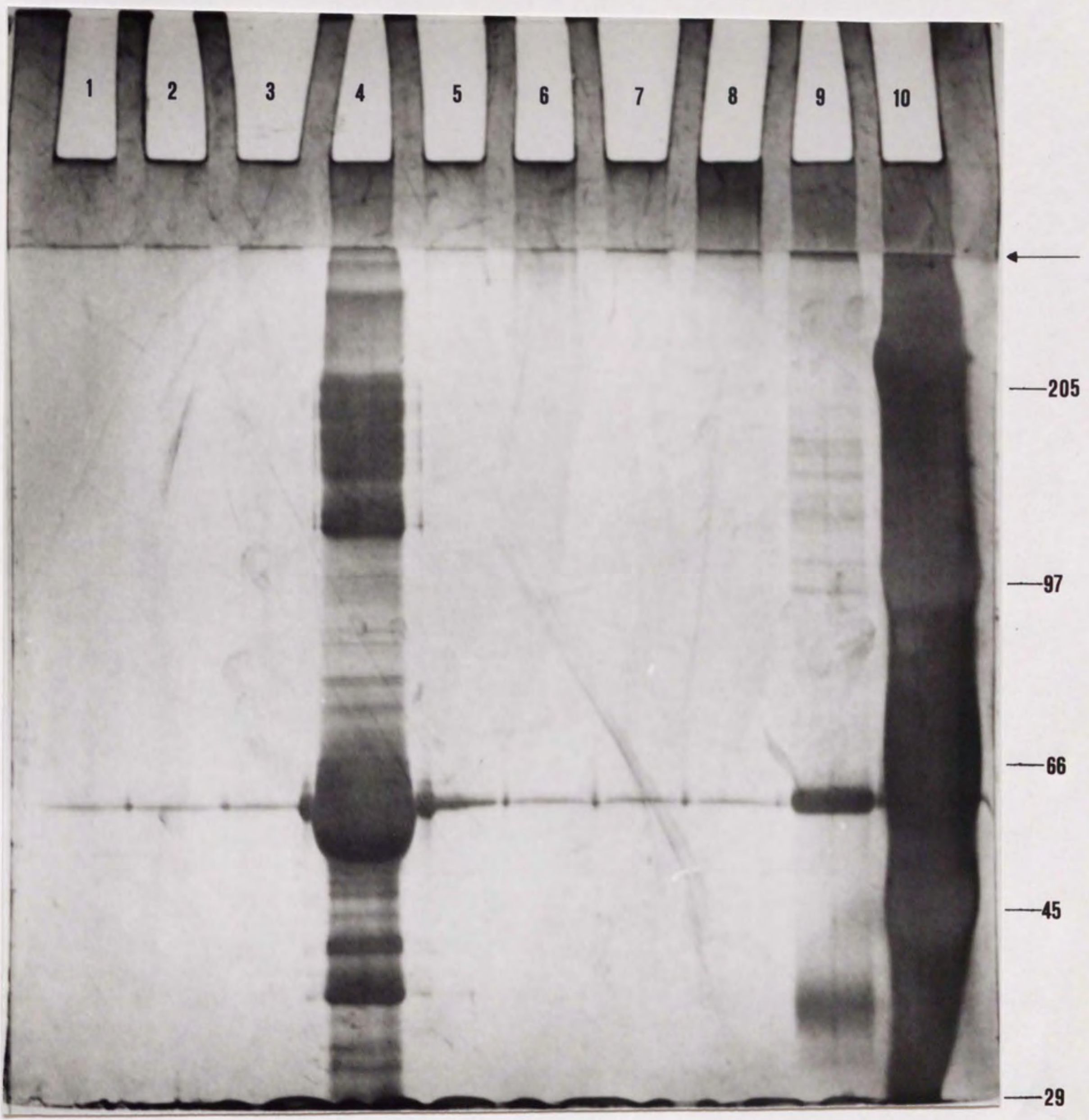
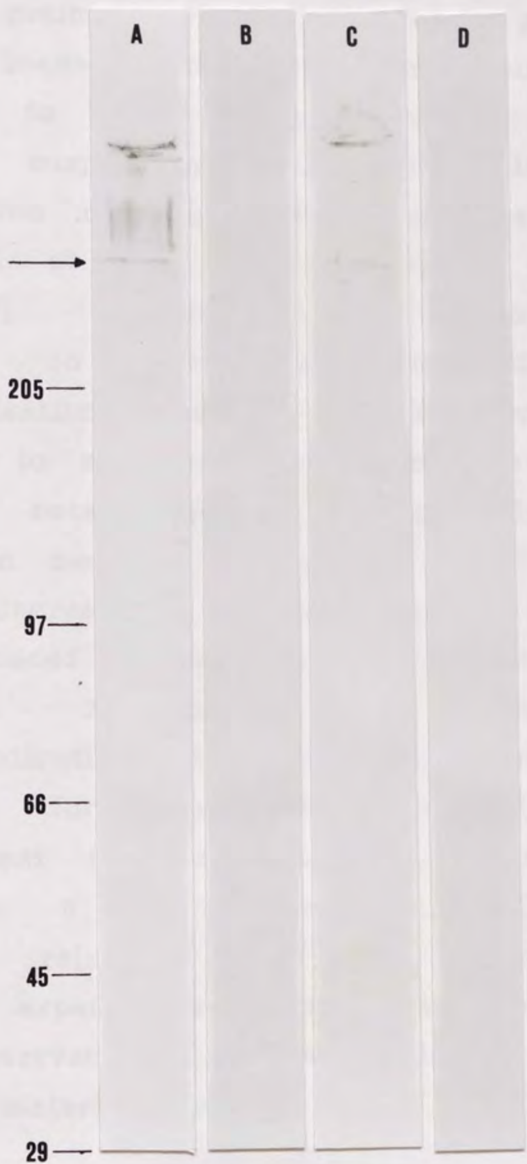


Figure 5.7

Protein blotting of purified unlabelled rat gastric mucin and incubation medium from a suspension of cells stimulated with 50μM-forskolin.



Results of protein blotting using unlabelled rat gastric mucin (lane A), unlabelled rat gastric mucin with preimmune serum (lane B), incubation medium (lane C) and incubation medium with preimmune serum (lane D). The arrow represents the division between the stacking gel (4% acrylamide) and the separating gel (8% acrylamide). The numbers and associated lines represent the molecular mass (kDa) and the positions of the marker proteins myosin, phosphorylase b, bovine serum albumin, ovalbumin and carbonic anhydrase (which ran with the solvent front).

1989b), rabbit intestinal mucin (Mantle & Thakore, 1988) and rat intestinal mucin (Mantle et al., 1989) were all found on protein blot analyses to react with their respective purified mucins producing staining material either within or at the top of the stacking gel. In control experiments using appropriately diluted preimmune serum no bands appeared on a blot of a gel loaded with rat gastric mucin (Fig 5.7). When exposed to blots of gels loaded with material released by the suspension of gastric cells (5.2.3) the antibody preparation recognized bands at the top of the stacking gel and at the stacking gel / separating gel border (Fig 5.7). Staining within the stacking gel was only present on blots of purified mucin. During homogenization prior to purification 'native' mucin was exposed to shearing forces and this may have produced a greater heterogenicity of molecular mass than that found in secreted mucin and could explain the presence of immunoreactive material within the stacking zone of gels loaded with purified mucin but not secreted material. No staining was present in the separating gel, indicating the antibody preparation had little affinity for non-mucin material present in the supernatant fraction (Plates 5.1, lane 4 and Fig 3.3, a Superose 6 gel filtration profile of [^{3}H]-labelled material released into the supernant fraction). In control experiments using preimmune serum no staining was observed on protein blots of gels loaded with secreted material (Fig 5.7).

In conclusion, the similarity between the blots obtained with purified native rat gastric mucin and the supernatant fraction derived from suspension of stomach cells stimulated with 50μM-forskolin indicates that the antibody preparation appears to be able to recognise rat gastric mucin secreted in vitro. Furthermore non-mucin proteins present in the incubation medium were not recognized by the antibody preparation.

5.3.1.4 Immunohistochemistry

Experiments in this section of work were carried out in collaboration with a final year project student Miss J.M. Williams.

Some medium-sized cells showed intense fluorescence, parietal cells (clearly distinguishable from their large size) showed no staining as did a population of smaller cells which probably included chief cells (Plate 5.2).

Polarization of the fluorescence was apparent in some, but not all, of the fluorescent cells (Plate 5.3). What appear to be granular structures can be seen at the apex of the cell. In a control experiment, incubation of the smear with FITC-labelled secondary antibody only gave negligible immunofluorescence.

The variation in polarization observed with the immunofluorescent cells could reflect different populations of mucus secreting cells. Cells from near the top of the gastric pit (surface, foveola and isthmus mucous cells) for instance, may be more likely to exhibit an apical accumulation of mucin granules. Alternatively, this effect could simply be the result of cell orientation on the smear itself. The antibody preparation, if specific for rat gastric mucin, would have been expected to recognize only those parts of the mucous cell which contained mucous granules. The above results are compatible with such specificity.

Immunoperoxidase labelling of 5μm paraffin-embedded stomach tissue sections showed that immunoperoxidase labelling (Plate 5.4) was heaviest in the pits and on the surface of the fundic epithelium. At a higher magnification (Plate 5.5) it can be seen that material inside the cells lining the pit was recognized. Replacement of the primary antibody with an equivalent dilution of preimmune serum reduced the immunoperoxidase labelling in the pits and on the

Plate 5.2

Immunofluorescence labelling of an isolated rat stomach cell smear. Clearly visible are parietal cells(p), smaller non-fluorescent cells (chief cells?;c) and mucous cells(m).Bar represents 10μm.

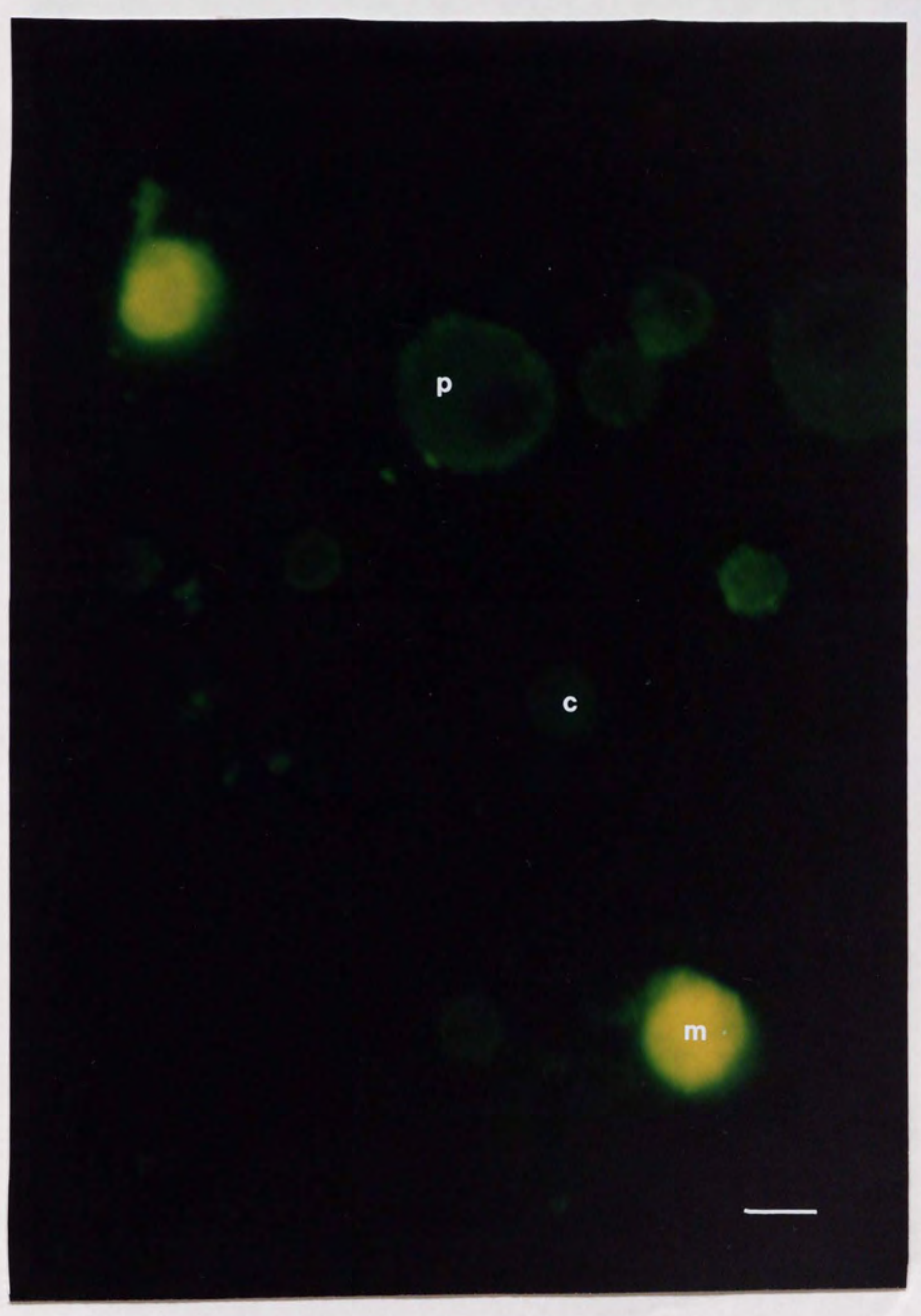


Plate 5.3

Immunofluorescence labelling of an isolated rat stomach cell smear. Clearly visible are polarized fluorescent positive cells. Bar represents 10μm.

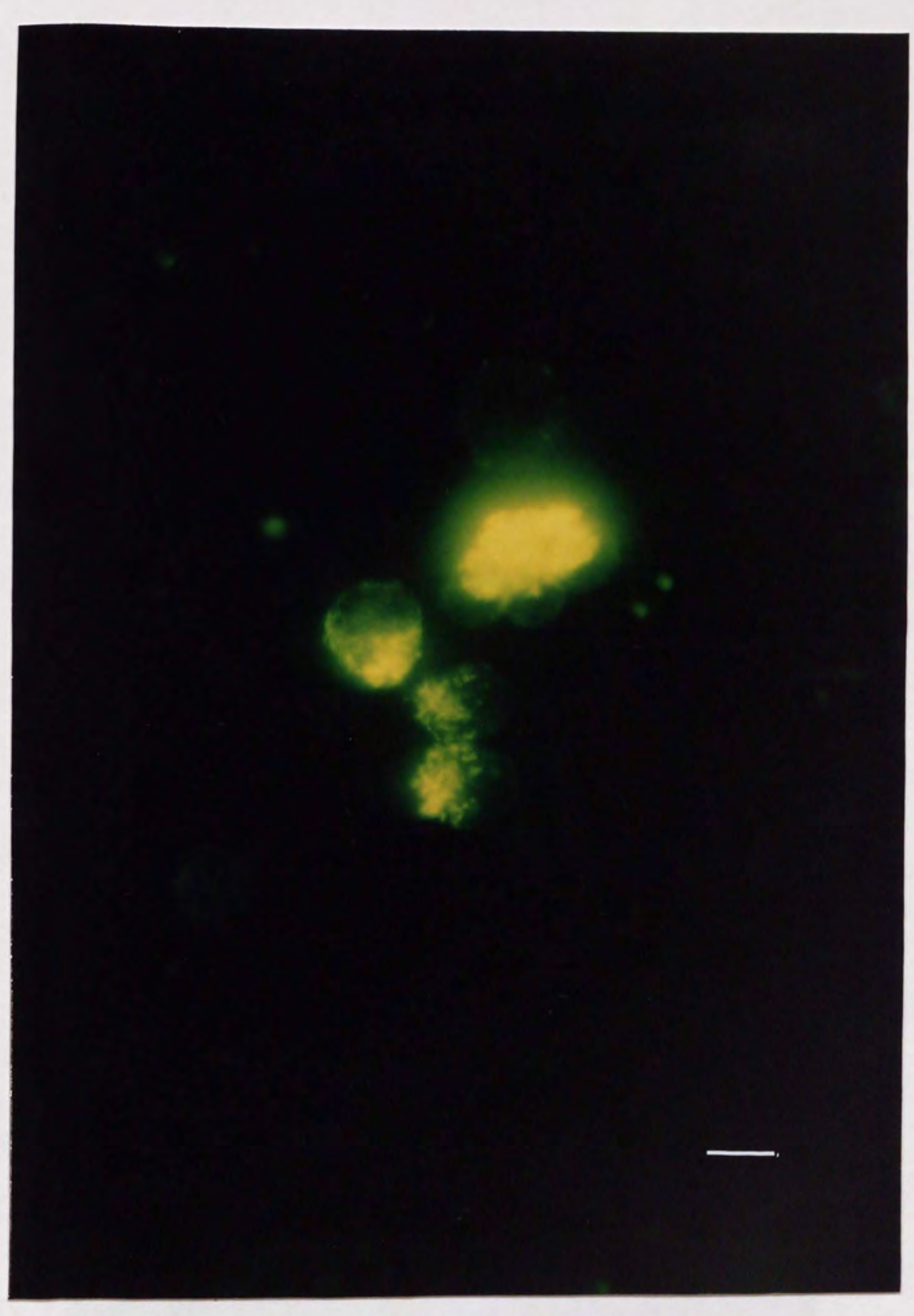


Plate 5.4

Immunoperoxidase labelling of 5μm paraffin embedded sections of rat fundic mucosa incubated with anti-rat 'native' gastric mucin antibody preparation.

Bar represents 50μm.

Plate 5.5

Immunoperoxidase labelling of 5μm-paraffin embedded sections of rat fundic mucosa incubated with anti-rat 'native' gastric mucin antibody preparation.

Bar represents 20μm.

surface to background levels (Plates 5.6 & 5.7). Similar results were obtained when primary antibody was preincubated with excess antigen for 24h (Plate 5.8). When primary antibody or secondary antibody or ExtrAvidin or hydrogen peroxide in substrate were omitted, only background labelling was observed. In summary, it seems likely that the antibody preparation is reasonably specific for mucin and mucous secreting cells.

5.3.1.5 Assessment of tissue specificity by indirect competitive solid-phase enzyme immunoassay

As the gastric mucin antibody preparation was to be used in solid phase EIA to quantitate small amounts of secreted mucin, it was important to assess its specificity within such a system. A sample (40µl) of gastric mucosal homogenate with a protein concentration of 9.4µg/ml was sufficient to reduce binding of antibody to 50% of maximum (B50), in an indirect competitive solid-phase EIA (Figure 5.8), using the final assay conditions described in 5.3.2.8. This suggests a mucin content of approximately 125ng/µg gastric homogenate protein. (c.f. B_{50} data 5.3.2.8). A sample (40µl) of jejunal mucosa homogenate with a protein concentration of 8.88mg/ml had little effect upon antibody binding to the ELISA plate. In control experiments, samples (40µl) of homogenate preincubated without antibody preparation gave no absorbance on the ELISA plate, thus indicating little non-specific interaction of homogenate material with the plate.

These results suggest that the purified rat gastric mucin antibody preparation can distinguish between gastric and small intestinal mucin and indeed does not recognize any soluble jejunal protein in this assay system. Mantle & Thakore, (1988) found the B_{50} values for intestinal and colonic homogenates were at least 27 times lower than for an equivalent stomach homogenate in an enzyme immunoassay using an antibody for rabbit small intestinal mucin. The present

Plate 5.6

Immunoperoxidase labelling of 5µm-paraffin embedded sections of rat fundic mucosa incubated with preimmune serum.

Bar represents 50µm.

Plate 5.7

Immunoperoxidase labelling of 5μm-paraffin embedded sections of rat fundic mucosa incubated with preimmune serum.

Bar represents 20μm.

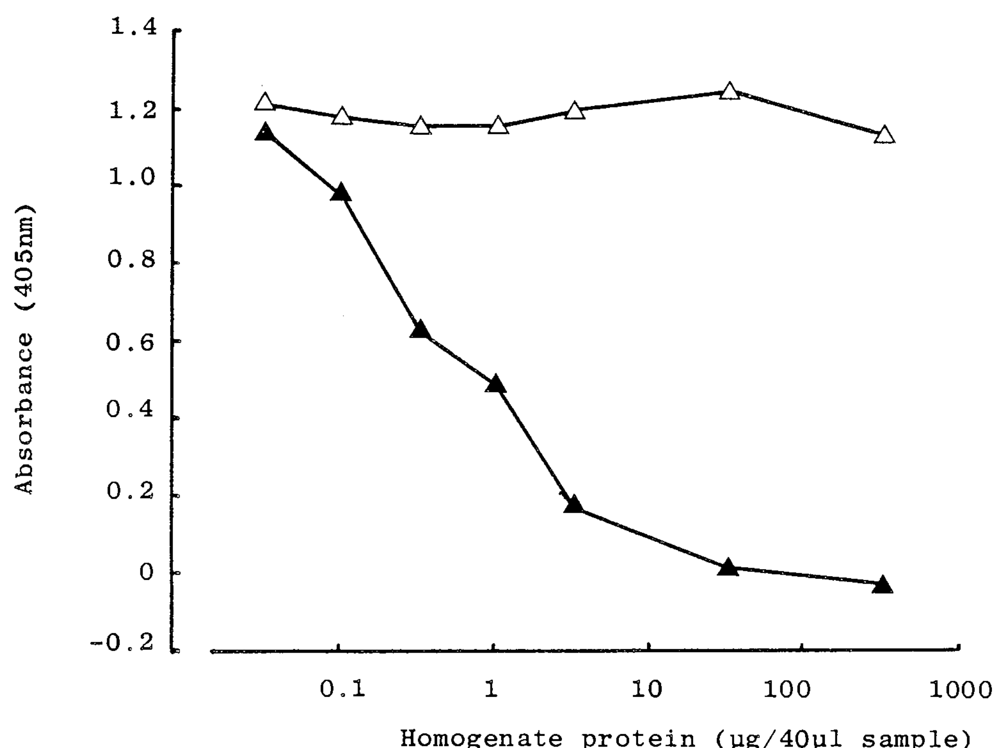
Plate 5.8

Immunoperoxidase labelling of 5μm-paraffin embedded sections of rat fundic mucosa incubated with preabsorbed anti-rat 'native' gastric mucin antibody preparation.

Bar represents 50μm.

Figure 5.8

Relationship between absorbance and the amount of homogenate of fundic or jejunal mucosa added to the indirect competitive solid-phase enzyme immunoassay.



antibody preparation appears to have an even greater ability to distinguish gastric from small intestinal homogenates.

In conclusion there is good evidence that the antibody preparation can detect gastric mucin both within mucous cells and after secretion into the incubation medium. Potentially perhaps the most likely non-mucin molecule that the antibody preparation might recognize is proteoglycan. The absence of any staining of basement membrane above background (Plate 5.4) and the tissue specificity of the EIA make this unlikely. Thus no evidence that the antibody preparation recognized non-mucin proteins was obtained, and it would therefore appear to be suitable for estimating mucin secretion from an isolated cell suspension using an indirect competitive solid-phase EIA.

5.3.2 Development and properties of an indirect competitive solid-phase enzyme immunoassay for 'native' rat gastric mucin

5.3.2.1 Assessment of optimal assay conditions by 'checker board' ELISA

Before the gastric mucin antibody preparation could be used in an indirect competitive solid-phase EIA, it was necessary to establish the optimal assay conditions. The optimal concentration of mucin and the appropriate dilution of the antibody preparation was determined by a 'checker-board' ELISA (Tijssen, 1985; Table 5.6). The antigen/antibody combinations fell into three classes. Firstly (Table 5.6, top left-hand corner) certain results were limited by the amount of protein A - alkaline phosphatase conjugate added i.e. no change in absorbance was observed with increasing antibody or mucin concentration. Secondly, (Table 5.6, bottom left-hand region) certain were limited by the amount of antibody only i.e. increasing the antigen added to the well did not increase absorbance. Lastly,

Table 5.6

'Checker-board' ELISA for antiserum against 'native' mucin

Final antibody preparation dilution	Nanograms antigen per well										
	6400	3200	1600	800	400	200	100	50	25	12.5	6.25
1:100	2.250	2.273	2.249	2.333	2.333	2.422	2.493	2.401	1.785	0.854	0.440
1:200	2.453	2.385	2.374	2.529	2.496	2.534	2.631	2.063	1.166	0.631	0.341
1:400	2.329	2.331	2.323	2.419	2.420	2.422	2.043	1.257	0.744	0.375	0.177
1:800	2.337	2.347	2.313	2.376	2.369	1.817	1.349	0.839	0.437	0.234	0.094
1:1600	1.705	1.773	1.913	1.950	1.784	1.287	0.789	0.490	0.259	0.106	0.030
1:3200	0.844	0.969	1.020	1.056	0.990	0.736	0.477	0.292	0.164	0.064	0.019
1:6400	0.438	0.523	0.545	0.551	0.506	0.435	0.304	0.195	0.117	0.043	0.000

Results are presented as absorbance at 405nm (-blank).

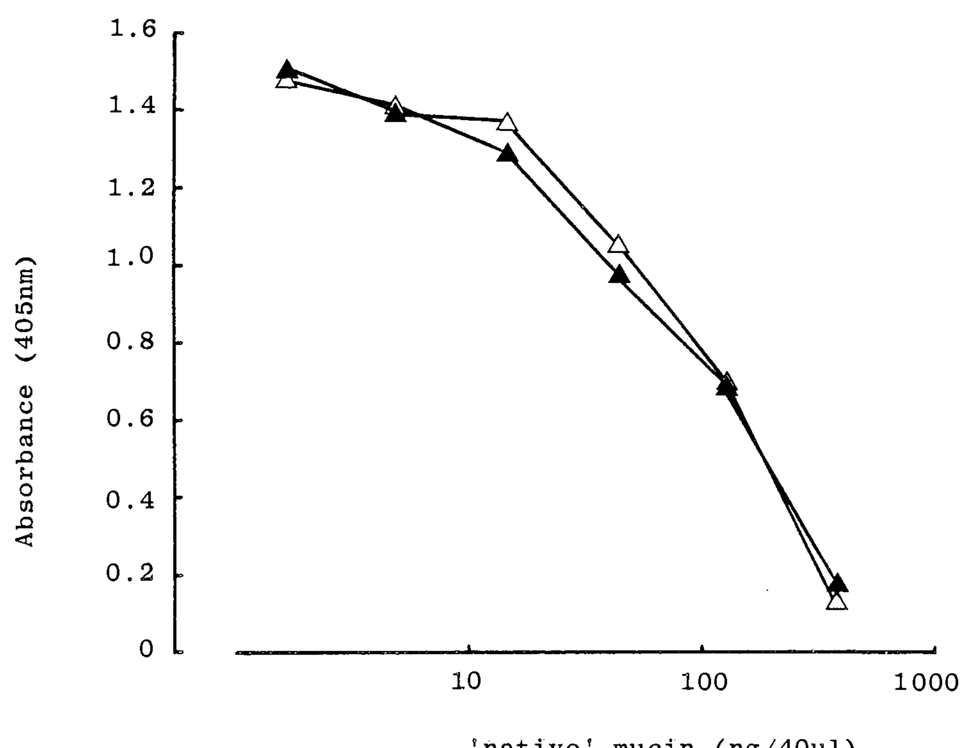
some combinations were limited by both the dilution of the antibody preparation and by mucin concentration i.e. changing the antibody or mucin concentration changed the absorbance. The choice of antibody dilution and mucin concentration for use in the indirect competitive solid-phase EIA was governed by three factors. Combinations giving absorbance values between 0.8 and 1.3 were desirable since microtitre plate photometer efficiency is then maximal (Tijssen, 1985). The highest dilution of antibody preparation showing the required absorbance should be used, to prevent unnecessary wastage. Similarly the lowest concentration of purified rat gastric mucin should be used for the same reason. Following these criteria the combination chosen for the indirect competitive solid phase EIA was 400ng purified rat gastric mucin (to coat the ELISA plate) and a 1:1600 dilution of the antibody preparation (this becomes 1:3200 on the ELISA plate since a 2-fold dilution occurs on mixing with the standard mucin or test samples).

5.3.2.2 Comparison of standard curves for the EIA with mucin standards dissolved in medium B' or PBS

The main objective of developing the EIA was to quantitate the secretion of gastric mucin by a suspension of isolated stomach cells into a medium containing Eagles minimum essential medium, 20mM-HEPES, 1mM-glutamine, 50μg/ml gentamicin and 1mg/ml BSA (medium B'). In initial experiments, the preincubation period between the antibody and the antigen was for 2h at room temperature. Standard gastric mucin dissolved in medium B' gave an EIA curve that was superimposable upon that obtained when standard mucin was dissolved in PBS (Fig 5.9). This result demonstrated that it was possible to add samples of medium used in incubating isolated cells directly to the assay without any interference resulting from the components of the incubation medium. Time-consuming buffer exchange or purification procedures were thus unnecessary. In

Figure 5.9

The effect of substituting medium B' for phosphate buffered saline in the preincubation step upon the relationship between absorbance and the amount of mucin added to the indirect competitive solid-phase enzyme immunoassay.



▲ medium B'

△ phosphate buffered saline

Preincubation for 2h at room temperature.

subsequent experiments involving isolated cell suspensions, samples were added to the preincubation plate without modification.

5.3.2.3 The effect of varying preincubation conditions on the standard curve of the EIA

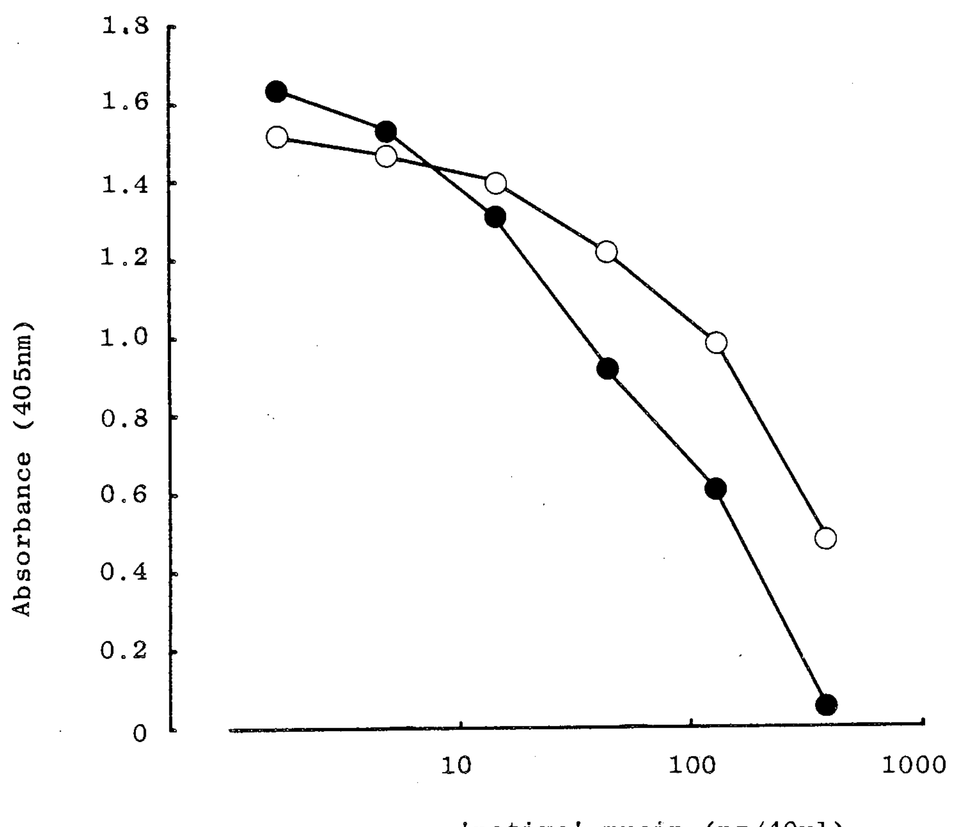
Preincubation of the antibody preparation and antigen at 37^0C overnight as opposed to 2 h at room temperature changed the shape of the standard curve (Fig 5.10). Three features are of note. Firstly the standard curve is much more linear. Secondly, there is increased sensitivity (slope) at low mucin concentrations and thirdly the overall sensitivity as measured by the B_{50} value is increased (Table 5.7). A possible explanation of the shape of the standard curve using preincubation for 2 h at room temperature is that these conditions were insufficient for equilibrium between the antibody preparation and the competing antigen to be established. Indeed, if a molecular mass of 2×10^6 is assumed then 10ng of mucin in a final preincubation volume of 80µl is at a concentration of 63pM so a kinetic limitation on the assay is not improbable. Since greater assay sensitivity was demonstrated with an overnight preincubation period at 37^0C this procedure was used routinely in all subsequent experiments.

5.3.2.4 The effect of protease inhibitors on the solid phase assay standard curve

Proteolysis of mucin during overnight preincubation at 37^0C of samples derived from cell suspensions was likely, and the inclusion of protease inhibitors at this stage was therefore desirable. Use of an antibody preparation containing 1µg/ml pepstatin, 1µg/ml leupeptin, 5mM-EDTA, 0.1mM-PMSF and 0.02% (w/v)sodium azide in the EIA gave a meaned standard curve that was superimposable upon that produced in the absence of protease inhibitors (Fig 5.11). In conclusion, protease inhibitors could be included in

Figure 5.10

The effect of altering the preincubation period from 2h at room temperature to overnight at 37°C upon the relationship between absorbance and the amount of mucin added to the indirect competitive solid-phase enzyme immunoassay.



○ 2h at room temperature

● overnight at 37°C

Table 5.7

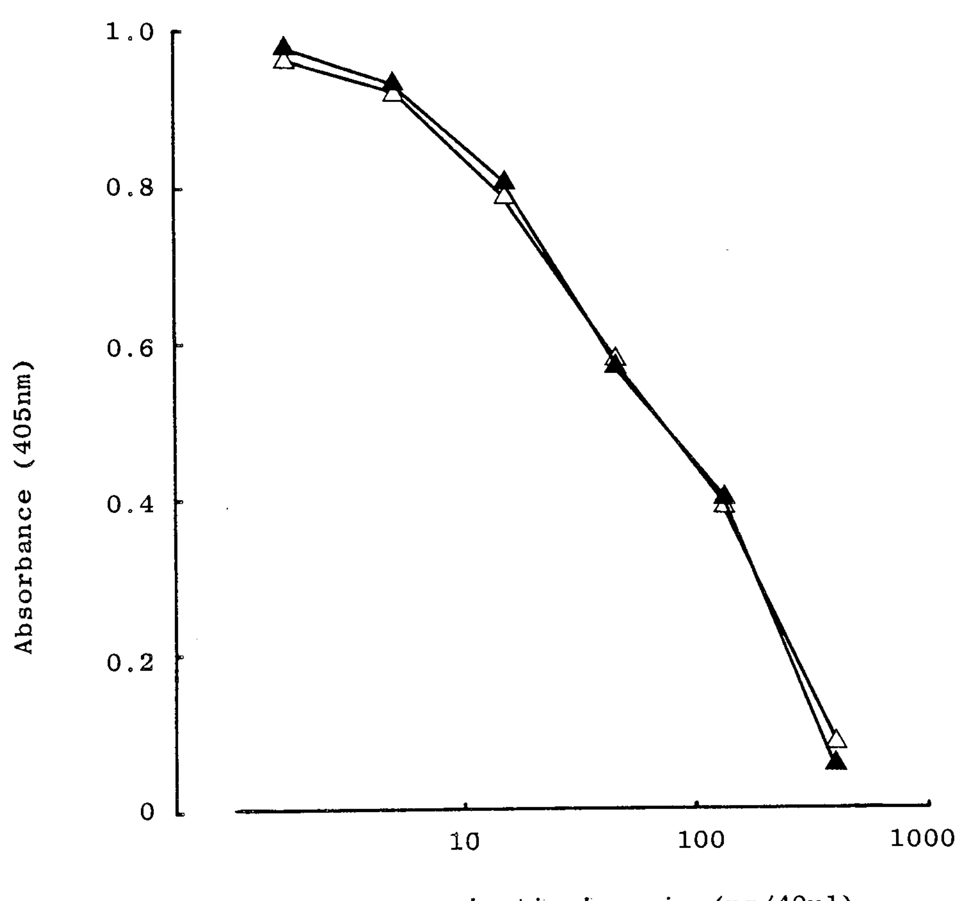
Effect of preincubation conditions on the B_{50} values obtained with EIA-1

Preincubation time and temperature	B_{50} (ng/40µl sample)
2h: Room temperature	89±12 (n=5)
Overnight: 37^0C	54±2 (n=6)**

** $p<0.01$ for effect of incubation conditions upon the B_{50} value obtained with EIA-1 (unpaired t-test).

Figure 5.11

The effect of preincubation in the presence or absence of protease inhibitors upon the relationship between absorbance and the amount of mucin added to the indirect competitive solid-phase enzyme immunoassay.



▲ protease inhibitors present

△ protease inhibitors absent

The standard curves presented here were from two different experiments and have been normalized so that a sample with no competing antigen has an absorbance value of 1.0. The original value in the presence of protease inhibitors was 1.359 $\pm$ 0.047 (n=12) and 1.482 $\pm$ 0.158 (n=4) in their absence.

the EIA without affecting the shape of the standard curve and were therefore used in all future experiments.

5.3.2.5 The effect of preincubation in the presence of 0.25mM-dithiothreitol on the solid-phase assay standard curve.

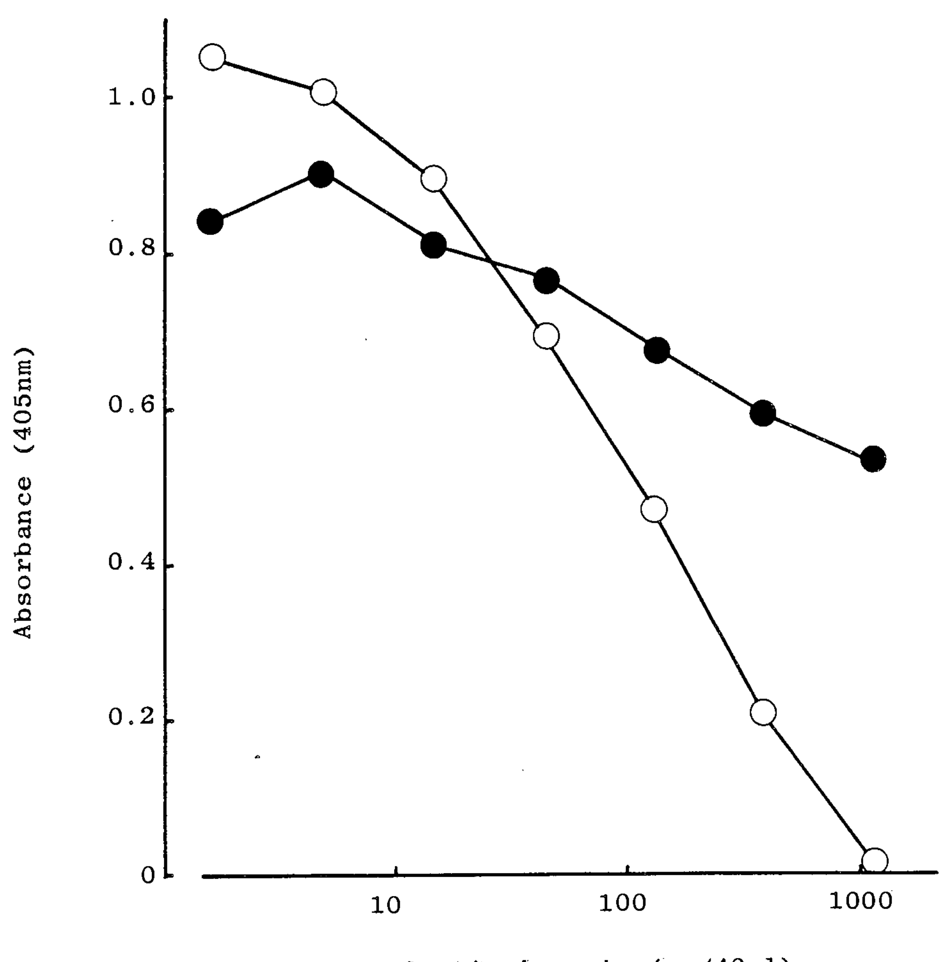
Preincubation of the antibody preparation and competing antigen containing 0.5mM-dithiothreitol (i.e. 0.25mM-dithiothreitol on preincubation plate) decreased the slope of the standard curve (Fig 5.12). These results are consistent with a change in the structure of the antigen such that antigenicity is lost (see 5.3.2.7). The relatively small effect of 0.5mM-dithiothreitol on the absorbance values obtained in the absence of competing antigen suggests that the antibody preparation was largely unaffected by preincubation in the presence of dithiothreitol. Since 0.5mM-dithiothreitol interfered with the assay this component was omitted from the medium used to incubate cells in all further experiments.

5.3.2.6 The effect of dilution of medium isolated from cells stimulated with 50µM-forskolin on results obtained with the EIA

The 1:3 serial dilution curve for the supernatant sample of incubation medium could be superimposed upon the standard curve (Fig 5.13). The antibody preparation therefore shows a similar affinity for epitopes which it recognizes on purified rat gastric mucin and on mucin present in the medium of cell suspensions. Protein blotting experiments (5.3.1.2) suggested differences in molecular mass between purified rat gastric mucin and material released into the incubation medium. However, this discrepancy is not reflected in a difference in the affinity of the antibody preparation in the EIA.

Figure 5.12

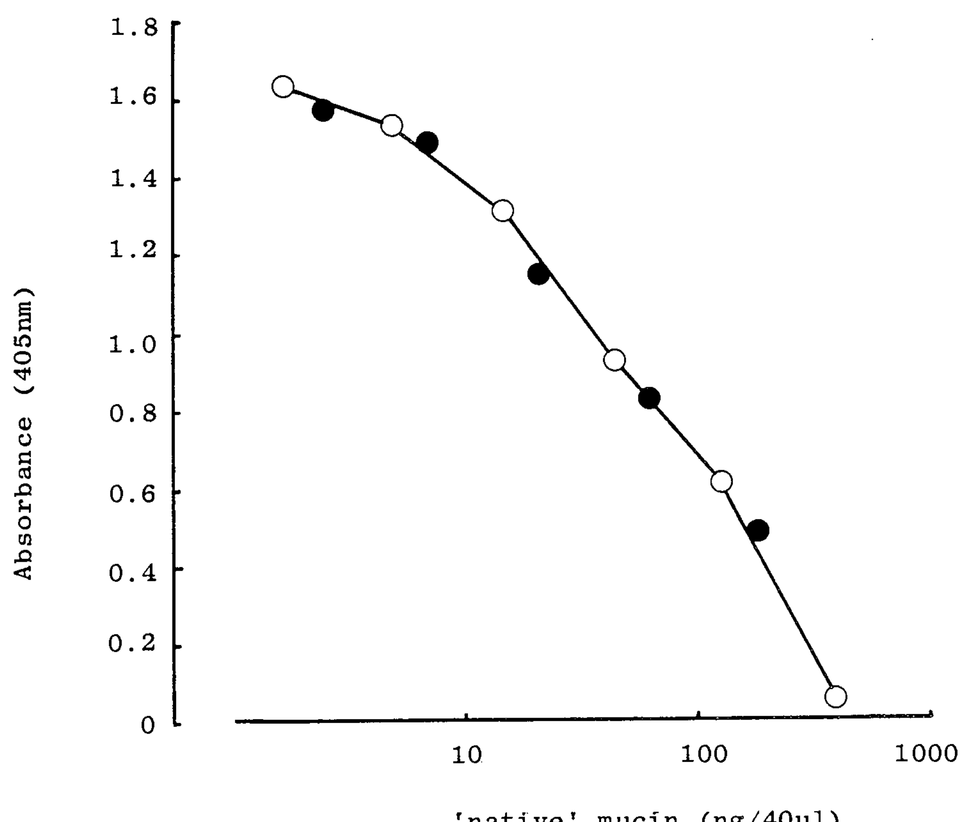
The effect of preincubation in the presence and absence of 0.5mM-dithiothreitol upon the relationship between absorbance and the amount of mucin added to the indirect competitive solid-phase enzyme immunoassay.



● 0.5mM-dithiothreitol present
○ 0.5mM-dithiothreitol absent

Figure 5.13

The effect of dilution of medium from a suspension of cells incubated with 50µM-forskolin for 60min upon the relationship between absorbance and the amount of mucin added to the indirect competitive solid-phase enzyme immunossay.



○ 'native' mucin

● serial 3-fold dilutions of incubation medium

Superimposition of the results obtained using incubation medium upon the standard curve has been performed by visual inspection.

5.3.2.7 The effect of modification of antigen structure on the standard curve of the EIA

Changes in the structure of an antigen can have one of three effects upon the assay standard curve. (i) the relative slope of the standard curve becomes zero i.e. antigenicity is completely abolished. (ii) the B_{50} value is increased i.e. the number of antigenic determinants is decreased but antibody affinity remains unaltered and (iii) no change in the relative slope or B_{50} value i.e. the number of antigenic determinants and the antibody affinity for them is unaltered.

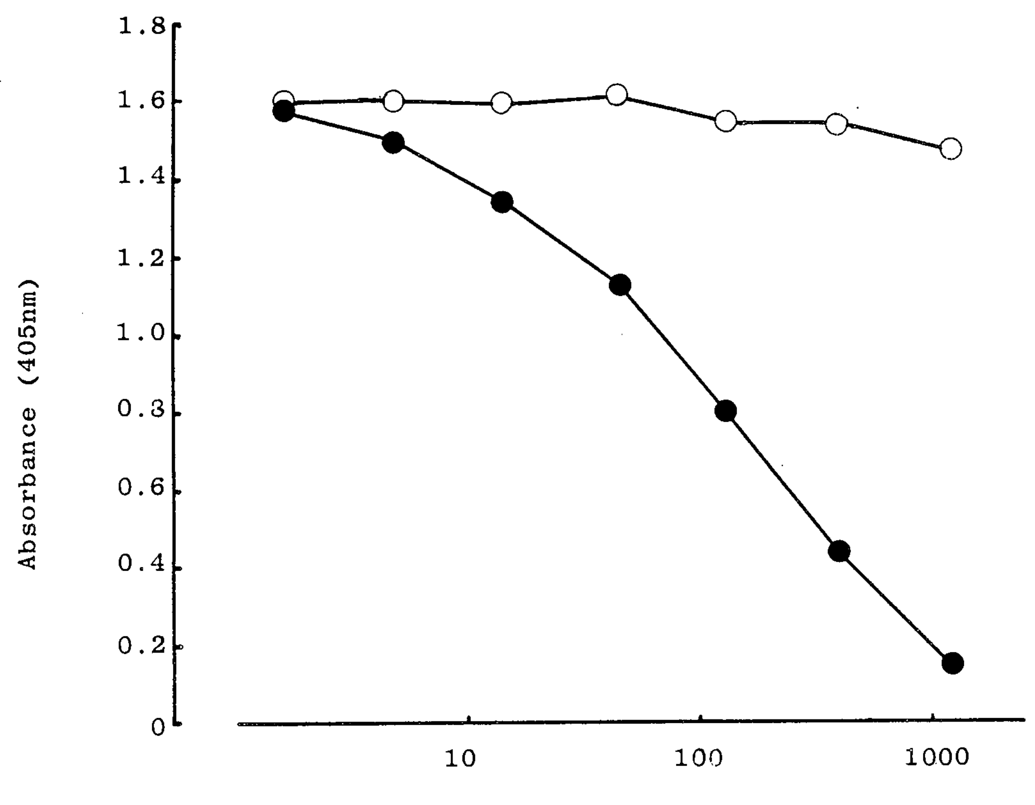
Preincubation of purified mucin with 0.1M-dithiothreitol completely destroyed its recognition by the anti rat mucin antibody preparation in the assay (Fig 5.14). Analysis of denatured rat mucin which had been reduced and carboxymethylated during isolation in the presence of 6M guanidine hydrochloride gave a similar result to that obtained with reduced 'native' rat mucin (Fig 5.15). Since reduction with dithiothreitol abolished the affinity of the mucin for the antibody preparation, the three-dimensional conformation of the antigen in its non-glycosylated regions, which are stabilized by disulphide bonds, would seem to be essential for recognition by the antibody preparation. Reduction of disulphide bridges will also destroy the polymeric structure of the mucin (Carstedt et al., 1985) but the results from protein blotting suggest that the molecular mass of mucin can vary without all or nothing changes in antigenicity.

Reduction of disulphide bridges in human intestinal mucin (Mantle et al., 1984) and rabbit intestinal and colonic mucin (Mantle & Thakore, 1988), also destroyed binding of antigen to the appropriate antibody.

Despite the high content of carbohydrate in the purified mucin (approximately 70-80% by weight), this does not appear to have acted as an antigenic

Figure 5.14

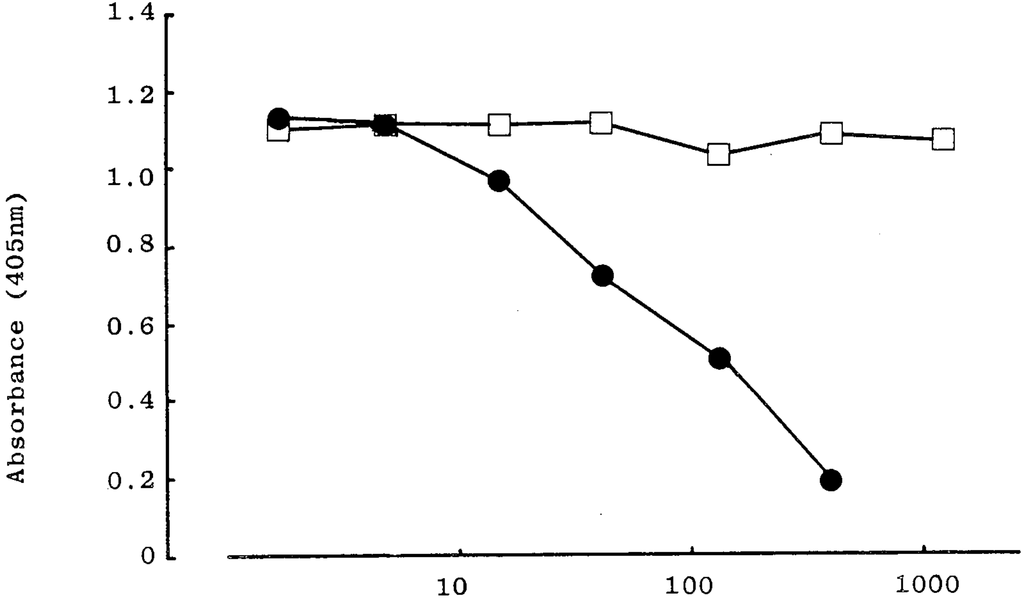
The effect of pretreatment with 0.1M-dithiothreitol upon the relationship between absorbance and the amount of 'native' mucin added to the indirect competitive solid-phase enzyme immunoassay.



● 'native' mucin
○ 'native' mucin pretreated with 0.1M-dithiothreitol

Figure 5.15

The effect of using reduced, carboxymethylated and denatured mucin upon the relationship between absorbance and the amount of 'native' mucin added to the indirect competitive solid-phase enzyme immunoassay.



● 'native' mucin

□ reduced, carboxymethylated and denatured mucin

determinant, since treatment with dithiothreitol would not have been expected to affect the antigenicity of such a component. Other workers have found similar results, for example, Qureshi et al., (1979) found that an antibody to human intestinal mucin did not recognise the ABH or Lewis blood group status of this mucin. Similarly, Mantle et al., (1984) observed that the same antibody would not bind to mono-, di-, tri- and oligo-saccharides typical of human small intestinal mucin.

In conclusion, the major antigenic determinant for the polyclonal anti-rat gastric mucin antibody preparation is probably in the non-glycosylated regions where interaction between mucin 'subunits' occurs. The antibody preparation probably recognizes epitopes created by the three dimensional folding of the mucin since procedures which disrupt the three dimensional structure either abolish or severely reduce binding in the EIA. The antibody preparation shows no apparent specificity towards the carbohydrate moiety of 'native' rat gastric mucin. One explanation of this is that there may be little difference between rat and rabbit in these structures which therefore are poorly immunogenic.

5.3.2.8 Indirect competitive solid phase enzyme immunoassay for native mucin: Final protocol and assay characteristics

The main aim of this section of work was to develop an indirect competitive solid phase EIA to quantitate secretion of gastric mucin from a suspension of rat stomach cells. The final assay protocol which was routinely used throughout further work (see Chapter 6) is shown schematically in Figure 5.5. The intraassay coefficient of variation was 6.2% (n=6) and the interassay coefficient of variation was 8.3% (n=4) (using samples obtained after 60 min incubation). The amount of competing purified mucin which produced half maximal absorbance (B_{50}) was 56±4ng (n=6). Henceforth this assay is referred to as EIA-1.

5.3.3 Development and properties of an indirect competive solid-phase enzyme immunoassay for 'reduced-denatured' mucin

5.3.3.1 Assessment of optimal assay conditions by 'checker-board' ELISA

The results for the 'checker-board' ELISA are shown in Table 5.8. Using the criteria for selecting the appropriate antiserum/antigen combination presented earlier (see 5.3.2.1), 800ng of 'reduced-denatured' gastric mucin was used to coat the ELISA plate and a 1:100 dilution of the antiserum preparation (final dilution on addition to standard mucin or test sample was 1:200) were chosen.

5.3.3.2 The effect of dilution of medium isolated from a cell suspension stimulated with 50μM-forskolin in the solid-phase assay

The curve for serial dilution of a sample of incubation medium (see 5.2.3.6) could be approximately superimposed upon the assay standard curve (Fig 5.16; c.f. Fig 5.13). Although the rabbit antiserum was prepared against reduced, denatured and carboxymethylated rat gasric mucin the epitopes it recognizes also seem to be present in secreted mucin and appear to be recognized with a similar affinity to the 'reduced-denatured' rat gastric mucin.

5.3.3.3 The effect of substituting native rat gastric mucin for 'reduced-denatured' rat gastric mucin in the EIA.

Further evidence that this antiserum preparation can recognize both 'native' and 'reduced-denatured' mucin with similar affinity comes from the finding that the standard curves were very similar whether 'native' or 'reduced-denatured' mucin was used as standard (Fig 5.17). N.B. in this assay the mucin bound to the ELISA plate was always 'reduced-denatured' mucin.

Table 5.8

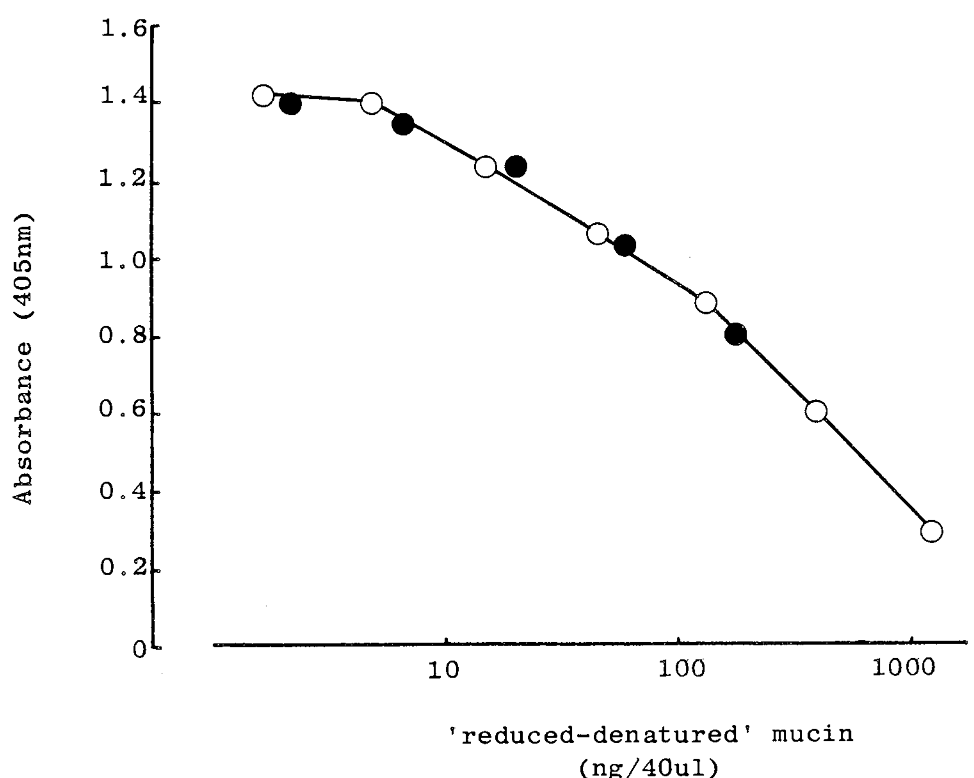
'Checker-board' ELISA for antiserum against 'denatured-reduced' mucin

Final antiserum dilution	Nanograms antigen per well										
	6400	3200	1600	800	400	200	100	50	25	12.5	6.25
1:100	1.808	1.624	1.557	1.410	1.298	1.213	0.947	0.691	0.486	0.344	0.258
1:200	1.179	1.121	1.117	1.003	0.777	0.699	0.551	0.341	0.199	0.141	0.127
1:400	0.733	0.665	0.645	0.587	0.491	0.434	0.355	0.270	0.202	0.148	0.119
1:800	0.378	0.344	0.338	0.293	0.244	0.198	0.158	0.115	0.071	0.047	0.035
1:1600	0.200	0.181	0.173	0.155	0.130	0.111	0.075	0.061	0.044	0.028	0.021
1:3200	0.103	0.106	0.098	0.095	0.076	0.066	0.045	0.036	0.029	0.017	0.018
1:6400	0.041	0.042	0.035	0.035	0.027	0.019	0.014	0.005	0.006	-0.003	-0.002

Results are presented as absorbance at 405nm (-blank).

Figure 5.16

The effect of dilution of medium from a suspension of cells incubated with 50µM-forskolin for 60min upon the relationship between absorbance and the amount of mucin added to the indirect competitive solid-phase enzyme immunoassay.



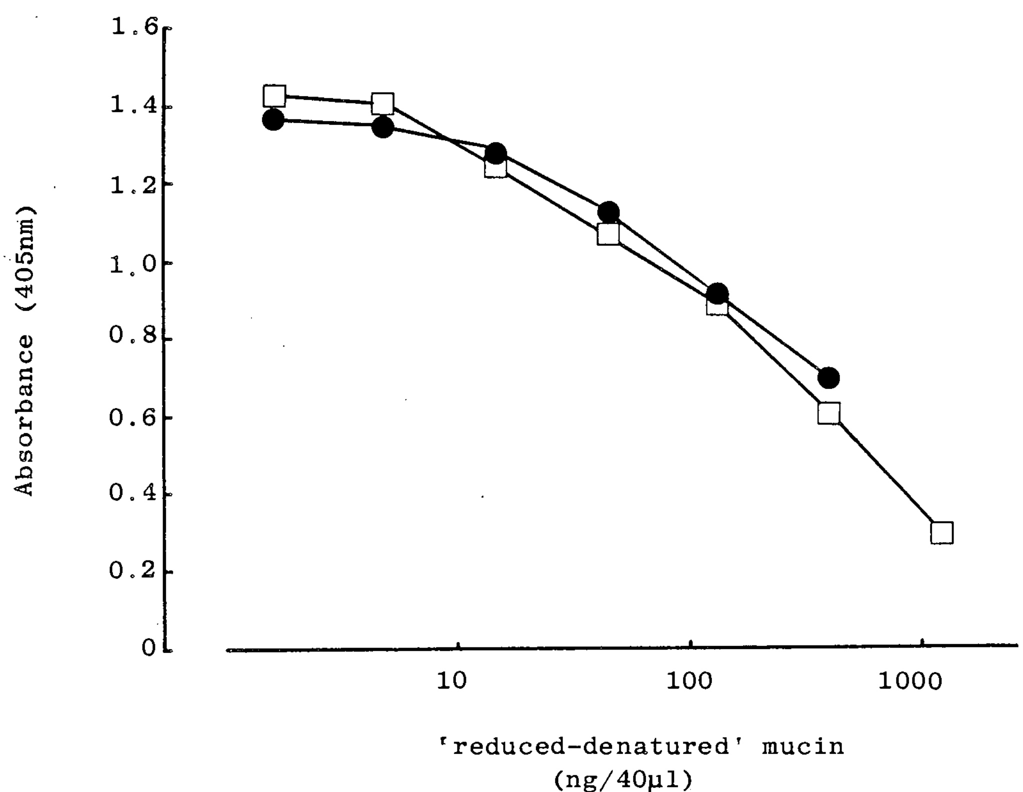
○ Reduced, carboxymethylated and denatured mucin

● Serial 3-fold dilutions of incubation medium

Superimposition of these results on the standard curve has been performed by visual inspection.

Figure 5.17

The effect of substituting 'native' mucin upon the relationship between absorbance and the amount of reduced, carboxymethylated and denatured mucin added to the indirect competitive solid-phase enzyme immunoassay.



□ Reduced, carboxymethylated and denatured mucin
● 'native' mucin

In conclusion, the antiserum preparation recognizes 'reduced-denatured' mucin and 'native' mucin. It also showed immunologically equivalent recognition of material in the supernatant fraction of an isolated stomach cell suspension. These properties should enable the antiserum to be used to quantitate mucin secreted from isolated stomach cell suspensions by solid-phase EIA. Also, since the antiserum specificity has been previously determined, it should prove useful in the further validation of the 'native' rat gastric mucin assay system (EIA-1).

5.3.3.4 Indirect competitive solid phase enzyme immunoassay for 'reduced-denatured' mucin: Assay characteristics.

The intraassay coefficient of variation was 13.7% (n=6). The interassay coefficient of variation was 15.3% (n=4) (using samples obtained after 60 min incubation). The amount of 'reduced-denatured' mucin required to reduce antibody binding to the ELISA plate by half (B_{50}) was 71±5ng per 40µl sample. Henceforth this assay is referred to as EIA-2.

5.4 GENERAL DISCUSSION

The primary aims of this section of work were the production of a rabbit polyclonal antibody preparation specific for rat gastric mucin and the development of a suitable solid-phase enzyme immunoassay capable of quantitating gastric mucin secreted by stomach cells.

To be usable the antibody preparation must not recognize non-mucin proteins to any large extent. It seems likely that the antibody preparation was specific for 'native' rat gastric mucin. Firstly, protein blotting experiments demonstrated that only high molecular weight material was recognized by the antibody preparation when purified rat gastric mucin or samples of incubation medium were analysed (Fig 5.7). Secondly, immunoperoxidase labelling was limited to mucous epithelial and mucous neck cells in sections of gastric mucosa (Plate 5.4). Thirdly, the inability of a jejunal homogenate to prevent the antibody preparation binding to an ELISA plate coated with rat gastric mucin, implies that the antibody preparation has little affinity for rat small intestinal mucin or other soluble jejunal proteins (Fig 5.8).

The fundamental requirements of the indirect competitive solid-phase EIA (EIA-1) are that mucin released into the incubation medium should be specifically recognized by the antibody preparation, and that the EIA should be sensitive to small changes in mucin concentration. The superimposition of results obtained by serial dilution of a sample of incubation medium on the standard curve (Fig 5.13) suggests that the antibody preparation had a similar affinity for epitopes present on both the standard and secreted mucin. The lack of recognition of 'reduced-denatured' mucin standard (Dekker et al., 1989b; Fig 5.15) and reduced mucin standard (Fig 5.14) in EIA-1 implies that the epitopes recognized by the antibody preparation are likely to be peptide rather than oligosaccharide in nature and require a specific three-

dimensional conformation. Finally, the low amount of standard mucin (approximately 50ng/40µl sample) required to reduce binding of the antibody preparation to the ELISA plate to half-maximal, implies that EIA-1 should be capable of detecting the small changes in mucin release that are likely to occur in cell suspension samples.

In conclusion, the results obtained in this section of work suggest that both EIA-1 and EIA-2 can be used to investigate the factors which affect the release of immunologically detectable mucin by cells isolated from the rat fundic mucosa.

5.5 SUMMARY

1. The polyclonal antibody preparation raised against purified 'native' rat gastric mucin exhibited specificity for the antigen using protein blotting, indirect immunofluorescence and immunohistochemical techniques. Furthermore it showed distinct tissue specificity upon analysis by indirect competitive solid-phase enzyme immunoassay.

2. The antibody preparation seemed to recognize polypeptide epitopes formed by the three-dimensional folding of the mucin rather than regions of polysaccharide.

3. An indirect competitive solid-phase immunoassay using the 'native' rat gastric mucin antibody preparation was developed, which could quantitate mucin secreted by isolated stomach cells. A similar assay was also developed using an antiserum preparation raised against 'reduced-denatured' rat gastric mucin.

Chapter Six

FACTORS WHICH AFFECT THE RELEASE OF IMMUNOLOGICALLY DETECTABLE MUCIN BY CELLS ISOLATED FROM THE RAT FUNDIC MUCOSA.

6.1 INTRODUCTION

6.1.1 Signal transduction pathways

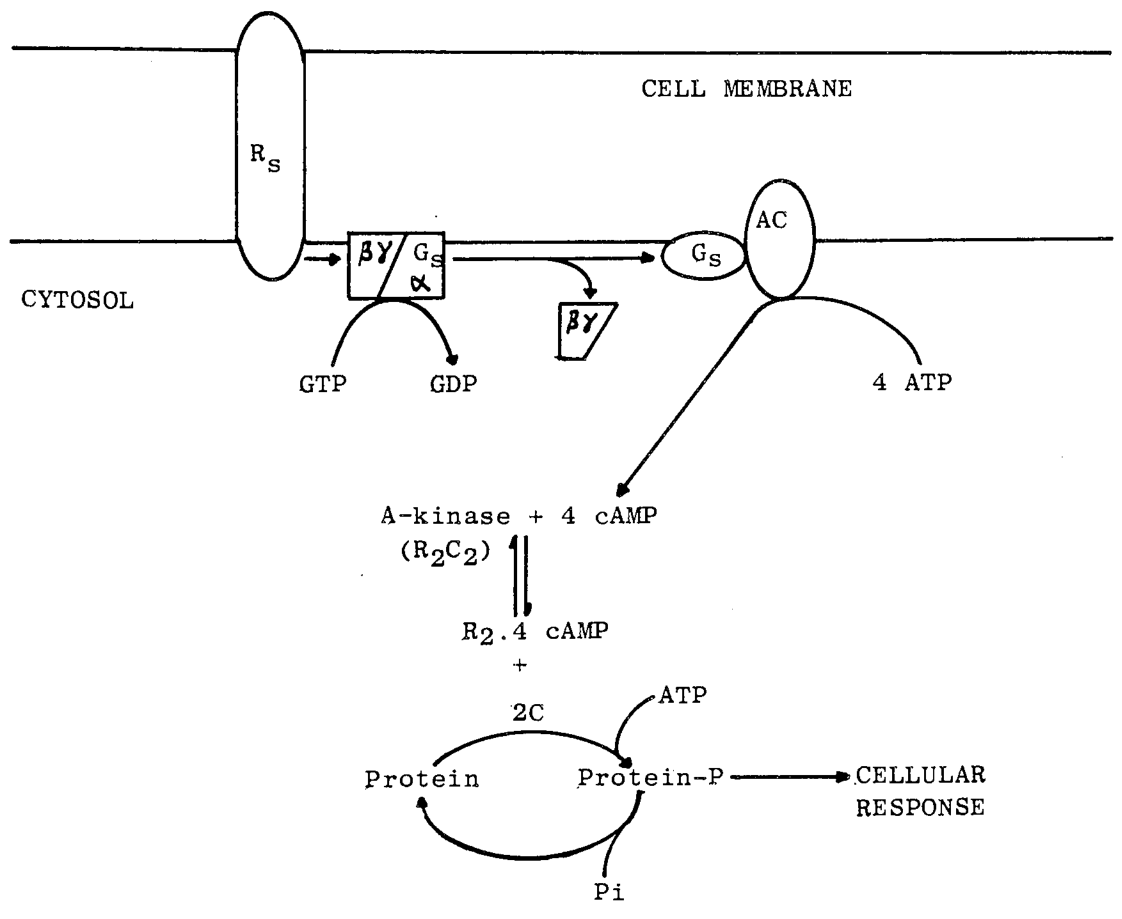
A common feature of all intercellular chemical signalling systems is that a mechanism must exist to transfer information across the plasma membrane of the target cell. One solution is for the signalling molecule to cross the membrane, another is for it to bind to specific recognition sites (receptors) upon the plasma membrane and to stimulate enzyme activity inside the cell. Thus, the chemical message delivered to the cell (the 'first messenger') is transduced at the plasma membrane into a rise in the intracellular concentration of the product of an enzymic reaction (the 'second messenger'). In this chapter the signal transduction pathways responsible for stimulating mucus secretion are investigated. Two major signal transduction pathways are now described.

6.1.1.1 The adenylate cyclase system

The first is the adenylate cyclase pathway, in which an increased formation of adenosine 3',5' monophosphate (cyclic AMP) from ATP leads to the activation of cyclic AMP-dependent protein kinase (protein kinase A). Adenylate cyclase is exclusively associated with the plasma membrane of target cells (Fig 6.1; Davoren & Sutherland, 1963) and appears to be a single polypeptide with a molecular mass of approximately 150 kDa (Smigel, 1986; Pfeuffer et al., 1985). Specific cyclic AMP phosphodiesterases catalyse the conversion of cyclic AMP to adenosine 5' monophosphate and hence terminate its messenger function. Inhibition of cyclic AMP phosphodiesterase activity with agents such as 3-isobutyl-1-methyl xanthine (Daley, 1982) can artificially increase intracellular cyclic AMP levels. A guanine nucleotide binding protein (G-protein) termed the stimilatory G-protein (G_S) is involved in the activation of adenylate

Figure 6.1

A schematic representation of signal transduction involving adenylate cyclase.



Key:

R_s	stimulatory receptor
AC	adenylate cyclase
$G_{s\alpha}$	α subunit of the stimulatory G-protein
βγ	βγ subunit of the stimulatory G-protein
A-kinase	cyclic AMP dependent protein kinase

cyclase by other signalling molecules. Inhibition of adenylate cyclase is mediated via an inhibitory G-protein belonging to the Gi family (Housley, 1987). Thus the intracellular concentration of cyclic AMP can rise or fall in response to a particular agonist.

The G_s protein has been shown to be a peripheral membrane protein consisting of α, ß and γ subunits, (Gilman, 1987). The binding of the hormone to its receptor increases the receptor's affinity for G_s. The interaction of the receptor with G_s allows the exchange of bound GDP for GTP on the α subunit ($G_{s\alpha}$-GTP) which is then thought to dissociate from the ßγ subunits. $G_{s\alpha}$-GTP then activates adenylate cyclase producing cyclic AMP (Fig 6.1). The signal is terminated when GTP bound to $G_s\alpha$ is hydrolysed to GDP (i.e. $G_{s\alpha}$ is a GTPase) and $G_{s\alpha}$-GDP dissociates from adenylate cyclase.

In the adenylate cyclase pathway the final steps are mediated by cyclic AMP-dependent protein kinase (protein kinase A; Walsh et al., 1968). In the absence of cyclic AMP protein kinase A is inactive and exists as a tetramer of two regulatory subunits (R) and two catalytic subunits (C). Each regulatory subunit has two binding sites for cyclic AMP and in its presence the inactive complex dissociates to leave a dimer of regulatory subunits and two free catalytic units (Hofmann, 1980). The free catalytic subunits modulate the activity of target proteins by phosphorylating serine or threonine residues in Arg-Arg-X-Ser-X sequences (where x denotes any residue) thus initiating the cellular response (Kemp et al., 1975). The termination of the cellular response is achieved by dephosphorylation of the phosphorylated substrates for protein kinase A and is catalysed by specific protein phosphatases (Ingebritsen & Cohen, 1983).

6.1.1.2 The phosphoinositide pathway

In this pathway, the binding of a chemical signalling molecule to its plasma membrane receptor leads to the activation of a specific phospholipase C (PLC) which hydrolyses the minor plasma membrane component phosphatidylinositol bisphosphate (PIP_2) (Berridge & Fain, 1979). Two 'second messenger' molecules: D-inositol 1,4,5 trisphosphate (IP_3) and 1,2-sn-diacylglycerol (DAG) are produced. A G-protein (putatively called G_p) may serve as an intermediary between the activated receptor and PLC (Sasaguri et al., 1985).

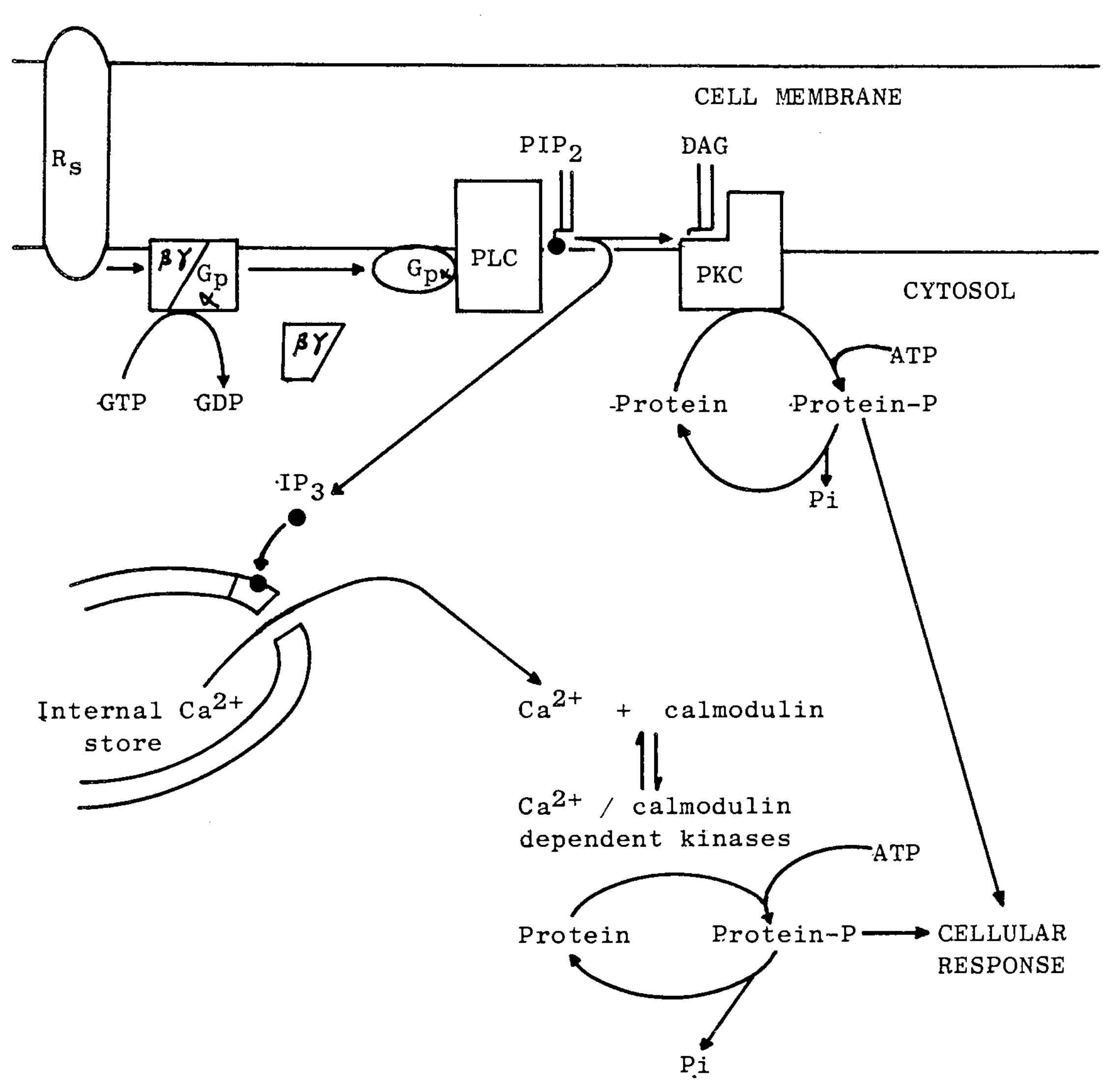
IP_3 mobilizes Ca^{2+} from an intracellular store (Fig 6.2), probably a subfraction of the endoplasmic reticulum (Suematsu et al., 1985). The messenger function of IP_3 can be terminated by the removal of the phosphate group at the 5 position by a specific phosphatase. Alternatively, IP_3 can be phosphorylated to inositol 1,3,4,5 tetrakisphosphate (IP_4) which may influence Ca^{2+} influx across the plasma membrane (Hansen et al., 1986).

The intracellular calcium receptor which mediates most of the processes induced by a rise in intracellular Ca^{2+} is calmodulin. This protein is a single polypeptide chain of approximately 150 amino acids which contains four high-affinity Ca^{2+} binding sites (Babu et al., 1985). The binding of Ca^{2+} to calmodulin induces a large conformational change in its structure, which allows the Ca^{2+}-calmodulin complex to interact with target proteins and thereby alter their activity. Among the proteins regulated by Ca^{2+}-calmodulin complexess are specific protein kinases, and membrane transport proteins (Klee et al., 1986).

The other cleavage product of PIP_2 (Fig 6.2), DAG, remains associated with the plasma membrane and activates a Ca^{2+}-sensitive phospholipid-dependent protein kinase (protein kinase C) by causing its

Figure 6.2

A schematic representation of the phosphoinositide pathway.



Key:

R_s	stimulatory receptor
PLC	phospholipase C
PKC	calcium-sensitive phospholipid- dependent protein kinase
$Gp\alpha$	α subunit of the putative G-protein (G_p)
βγ	βγ subunit of the putative G-protein (G_p)
PIP_2	phosphatidylinositol 4,5 bisphosphate
DAG	1,2-sn-diacylglycerol
IP_3	inositol 1,4,5 trisphosphate

translocation from the cytosol to the membrane and reducing its requirement for Ca^{2+} (Nishizuka, 1984). DAG is rapidly metabolized either via diacylglycerol kinase to phosphatidic acid or via diacylglycerol lipase to free glycerol and its constituent fatty acids (commonly stearic in position 1 and arachidonic in position 2). Thus, DAG may also generate other messenger molecules such as prostaglandins (Holub, 1970).

Protein kinase C exists in several isoforms and 6 genes have now been identified (Nishizuka, 1988). The α, βI, βII and γ transcripts correspond to peaks III, II and I of enzyme activity isolated on a hydroxyapatite column (Kikkawa, 1987). It is not known which isoforms are present in rat gastric mucous epithelial cells, therefore the general term protein kinase C will be used throughout this section.

6.1.2 Pharmacological agents used to study signal transduction pathways

One of the major aims of this section of work was to establish whether the adenylate cyclase and/or the phosphoinositide signal transduction pathways were involved in the regulation of mucin secretion by rat gastric mucous epithelial cells. Both of these intracellular signaling pathways can be activated by the addition of specific pharmacological agents to intact cells.

6.1.2.1 Forskolin

Forskolin is an unusual plant diterpene isolated from the roots of Coleus forskohlii (Fig 6.3). Forskolin reversibly activates adenylate cyclase in membranes and intact cells (Metzger & Lindner, 1981; Seamon et al., 1981). Forskolin appears to interact with adenylate cyclase through a site present on the catalytic subunit (Seamon & Daly, 1981). Agonists

Figure 6.3

The structure of forskolin.

which act through G_s markedly potentiate effects of forskolin on the catalytic subunit (Insel et al., 1982).

6.1.2.2 Calcium ionophone A23187

A23187 belongs to a family of naturally occuring carboxylic ionophores (Reed & Lardy, 1972) and is extracted from the bacterium Streptomyces chartrensensis (Fig 6.4). A23187 is highly selective for divalent over monovalent cations (Pressman, 1976) and binds with Ca^{2+} to form a $(A23187^-)_2\ Ca^{2+}$ complex. It is a neutral charge carrier (i.e. it does not affect the membrane potential) and with the intracellular calcium concentration ($[Ca^{2+}]_i$) at 10^{-7}M and medium $[Ca^{2+}]$ at 1.8mM it will raise $[Ca^{2+}]_i$ probably by effecting a $2H^+$ for Ca^{2+} exchange. A23187 has an affinity for Ca^{2+} only three times greater than that for Mg^{2+} and can therefore exert profound effects on cellular Mg^{2+} levels under certain circumstances (Campbell & Siddle, 1976). Cellular Mg^{2+} levels are typically 0.5mM whilst medium Mg^{2+} levels are 0.8mM.

6.1.2.3 Phorbol-12-myristate-13-acetate (PMA)

PMA is a tetracyclic diterpene isolated from the seed oil of Croton tiglium (Fig 6.5). Although PMA is not intrinsically carcinogenic, it enhances the formation of tumours by subthreshold doses of carcinogenic agents and is called a tumour promoter (Hecker, 1968). The ability of PMA to promote tumours is probably related to its ability to activate protein kinase C. Thus the PMA receptor co-purified with protein kinase C (Leach et al., 1983). Indeed there is a structural resemblance between the acylation of positions 12 and 13 and diacylglycerol (Fig 6.5). The orientation of the hydroxyl group at position 4 is also important. In PMA it is in the ß position but if α the molecule is completely inactive as an activator of protein kinase C (Van Duuren et al., 1979). 4α-PMA can therefore be used as a negative control compound.

Figure 6.4

The structure of the calcium ionophore A23187.

Figure 6.5

The structure of phorbol-12-myristate-13-acetate.

A comparison of the structures of diacylglycerol (top) and phorbol-12-myristate-13-acetate (bottom). Phorbol-12-myristate-13-acetate contains a diacylglycerol-like moiety (dotted area) within its structure. R_1 and R_2 represent hydrocarbon chains of fatty acid.

6.1.3 Aims of this section

To use the indirect competitive solid-phase EIA to perform the following experiments.

1) The assessment of the time-dependence of rat gastric mucin secretion in the presence and absence of the adenylate cyclase activator, forskolin.

2) The use of forskolin, A23187 and PMA to investigate the signal transduction pathways utilized by rat gastric mucous epithelial cells to induce mucin secretion.

3) Establishment of the effect of concentration of secretin and isoprenaline on rat gastric mucin release.

4) A preliminary investigation into the effects of carbachol, 3-isobutyl-1-methyl xanthine (IBMX) and truncated glucagon-like peptide-1 (TGLP-1). Carbachol and IBMX have been discussed previously (1.4.2.2 & 6.1.1.1). Some brief information on TGLP-1 is in Appendix 3.

6.2 METHODOLOGY

6.2.1 Incubation of cells

Isolated rat fundic epithelial cells were prepared as described in 2.1 and preincubated for 3h as described in 3.2.2. except that no D-[6-^{3}H]glucosamine was added. Potential secretogogues in suitable solvents (6.2.4) were added as stock solutions directly to polyethylene scintillation vials (usually to a total volume of 10µl). An equivalent amount of solvent was added to control vials. Cell suspension (at 10^7 cells/ml) was added to each vial to give a final volume of 1.2ml. The air space in the vials was gassed with 95% 0_2/5% $C0_2$ and incubation was for 1h at 37^0C with shaking at 150 cycles/min. In experiments using EIA-1 upto 21 samples of incubation medium (9 treatments in duplicate + 3 controls) could be investigated and a typical experimental week is illustrated in Fig 6.6. In addition three portions (300µl; see 6.2.3) of cell suspension were removed immediately before the secretory period to determine the mucin content.

6.2.2 Preparation of incubation medium samples for EIA-1

After the incubation period, 1ml of cell suspension was removed from the scintillation vials, aportioned to 1.5ml microfuge tubes and centrifuged at 12000g for 30 s. A portion (900µl) of each superatant was added to 100µl of PBS containing 10µg/ml pepstatin, 10µg/ml leupeptin, 50mM-EDTA, 1mM-phenylmethylsulphonyl fluoride (PMSF) and 0.2% (w/v)-sodium azide. A portion of each sample (40µl) was then immediately preincubated with antibody preparation at 37^0C in a humid chamber overnight as described in 5.2.6. Further procedures were as described in 5.2.6. Unused sample material was frozen and stored at -20^0C.

Figure 6.6

Experimental protocol

MONDAY	TUESDAY	WEDNESDAY	THURSDAY	FRIDAY
Collate EIA data from previous week	Isolate & incubate cells.	Assay samples	Isolate and incubate cells.	Assay samples
Prepare for experiment 1.	Preincubate samples	Prepare for experiment 2.	Preincubate samples	
Block preincubation plate	Block ELISA plate	Block preincubation plate	Block ELISA	

6.2.3 Preparation of cellular samples for EIA-1

Samples of cell suspension required for the determination of intracellular mucin were removed prior to the secretory period. Cell suspension (3x300µl, i.e. 3 x $3x10^6$ cells) samples were placed in 1.5ml microfuge tubes and centrifuged as described in 6.2.2. Cell pellets were homogenized in 100µl of PBS containing 1µl/ml pepstatin, 1µg/ml leupeptin, 5mM-EDTA, 0.1mM-PMSF and 0.02% (w/v)-sodium azide using a microhomogenizer system (Biomedix, Pinner; Hearse, 1984). Homogenization was for 2 min at 2000r.p.m. Homogenates of cell pellets were diluted 1:150, 1:230 and 1:300 with the above buffer before 40µl of each solution was assayed as described in 6.2.2.

6.2.4 Preparation of stock solutions of PMA, 4α-PMA, Forskolin and A23187

Stock solutions of PMA or 4α-PMA were prepared in dry DMSO at a concentration of 100µM and aliquots were stored at -20^{0}C. On the day of the experiment the PMA (or 4α-PMA) stock was thawed at room temperature and diluted to the required concentration by serial dilutions in DMSO, and a small volume (usually 2µl) was added to incubation vials. DMSO was also added to the control vials so that the final concentration was 0.167% (v/v) in all vials.

A stock solution of forskolin (80mM) was prepared in dry DMSO and stored at -20^{0}C in an amber glass bottle. Subsequent procedures were the same as with PMA except that 2.5µl DMSO was usually added to incubation vials and the final concentration was 0.208% (v/v) in all vials. In experiments where both forskolin and PMA were added to incubation vials the final concentration of DMSO was 0.375% (v/v) in all vials.

A stock solution of the calcium ionophore A23187 (6mM) was prepared in dry DMSO and aliquots were stored at $-20^{0}C$. Subsequent procedures were as described for PMA. In experiments where A23187 and PMA were both investigated, the final concentration of DMSO was 0.333% (v/v) in all vials.

6.2.5 Preparation of secretin and isoprenaline stock solutions

Secretin and isoprenaline stock solutions were prepared as described previously (see Table 4.2).

6.2.6 Presentation of data

The effect of an agent upon immunologically detectable gastric mucin secretion was expressed by normalizing the data to % stimulation above basal release. This was calculated by the following expression:-

$$\%\ \text{stimulation above basal} = \frac{\left(\begin{array}{l}\text{ng mucin released}\\ \text{in presence of agent}\end{array} - \begin{array}{l}\text{ng mucin present}\\ \text{at t=0}\end{array}\right) - \left(\begin{array}{l}\text{ng mucin released}\\ \text{in absence of agent}\end{array} - \begin{array}{l}\text{ng mucin present}\\ \text{at t=0}\end{array}\right)}{\left(\begin{array}{l}\text{ng mucin released}\\ \text{in absence of agent}\end{array} - \begin{array}{l}\text{ng mucin present}\\ \text{at t=0}\end{array}\right)} \times 100$$

Normalization of the data enabled meaningful comparisons to be made between experiments despite variations in the absolute magnitude of the untransformed data. Both control and experimental measurements were always peformed on the same cell batch.

Unless stated otherwise the computer program FIT (Barlow, 1983) was used to estimate the EC_{50} of an agent.

6.3 RESULTS AND DISCUSSION

In this chapter discussion will be restricted in scope since a comparison of the two methods developed in this work and of results for the stomach with other regions of th gastrointestinal tract will be presented in the general discussion (Chapter 7).

6.3.1 Time-dependance of secretion of immunologically detectable gastric mucin from cells stimulated with 50μM-forskolin

The time-dependance of mucin secretion was assessed using both of the solid-phase EIA's (EIA-1 and EIA-2). The results will be presented in three sections: (i) the time-dependance of rat gastric mucin secretion measured using EIA-1 (antibody to 'native' mucin); (ii) the time-dependance of rat gastric mucin secretion measured using EIA-2 (antibody to 'denatured-reduced' mucin); and (iii) comparison of the results from the EIA-1 and EIA-2 systems. This will be followed by a section discussing the time-course data.

6.3.1.1 Time - dependance of secretion of immunologically detectable material measured using EIA-1

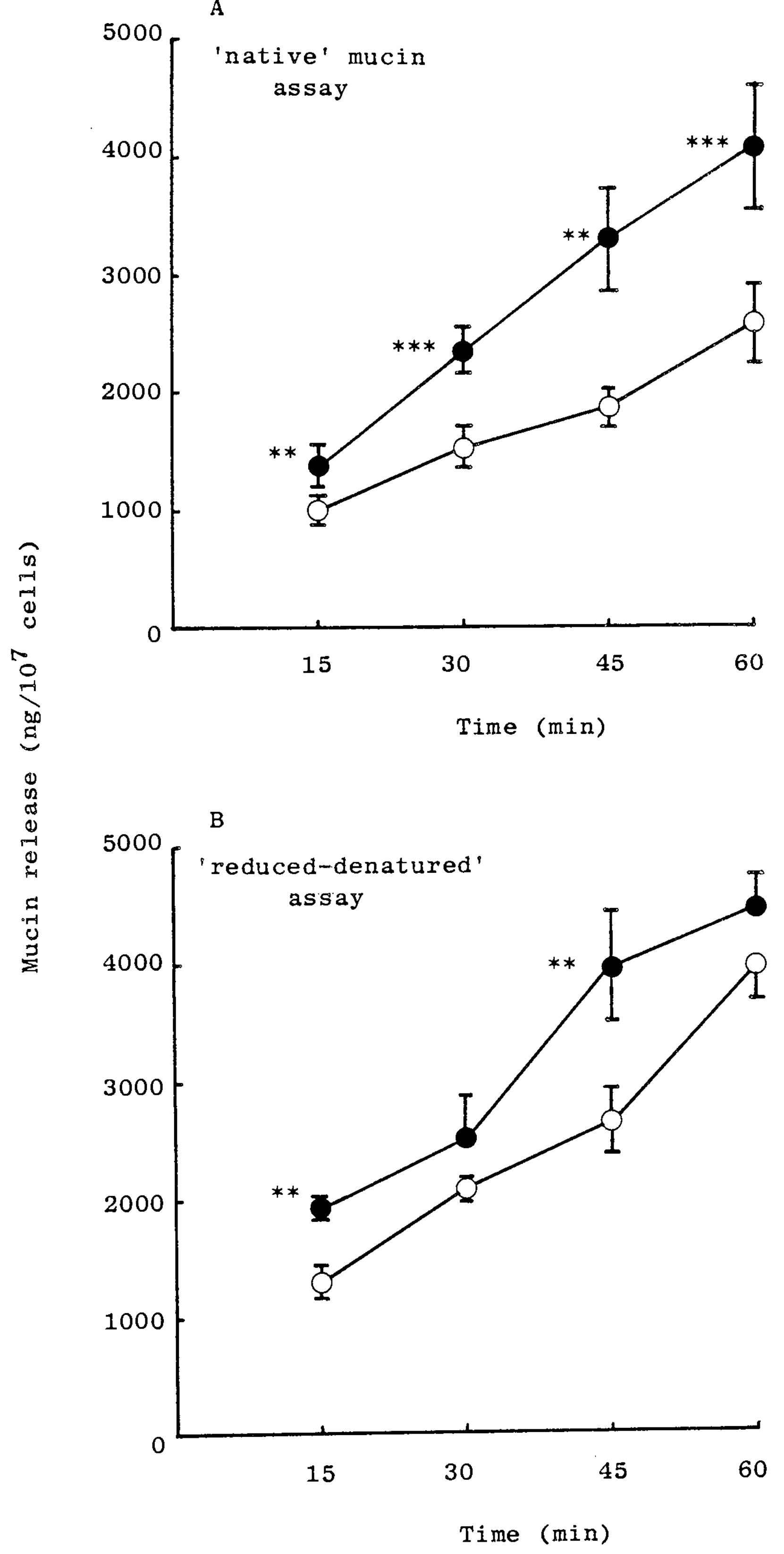
Release of immunologically detectable material into the incubation medium was essentially linear over 15-60 min under both control conditions and in the presence of 50μM-forskolin (Fig 6.7 A). At all time intervals tested 50μM-forskolin significantly stimulated the release of immunologically detectable material into the medium. The stimulation at 60 min represented a 57% increase over the basal release.

In a separate series of experiments, the cellular content of immunologically detectable material was 124±20μg / 10^7 cells (n=5), and in this series material

Figure 6.7

Effect of time on the release of mucin into the incubation medium. Results are presented as means ± S.E.M. from six batches of cells. $**P<0.01$, $***P<0.001$ for comparison of control (○) with 50μM-forskolin stimulated (●) result by paired t-test. Basal (t=0) mucin was 2462 ± 135ng/10^7 cells and 2180 ± 323ng/10^7 cells for A and B respectively (no significant difference). In A the equation for the control regression line is $y=465 + 34.34x$ and that for 50μM-forskolin is $y=485.5 + 61.29x$. In B the equation for the control regression line is $y=346.5 + 57.68x$ and that for the stimulated cells is $y=940.5 + 60.92x$.

Figure 6.7



released over a 60 min incubation period in the absence or presence of 50µM-forskolin represented 2.6±0.5% and 4.7±1.2% of the cellular material respectively.

6.3.1.2 Time-dependance of secretion of immunologically detectable material measured using EIA-2

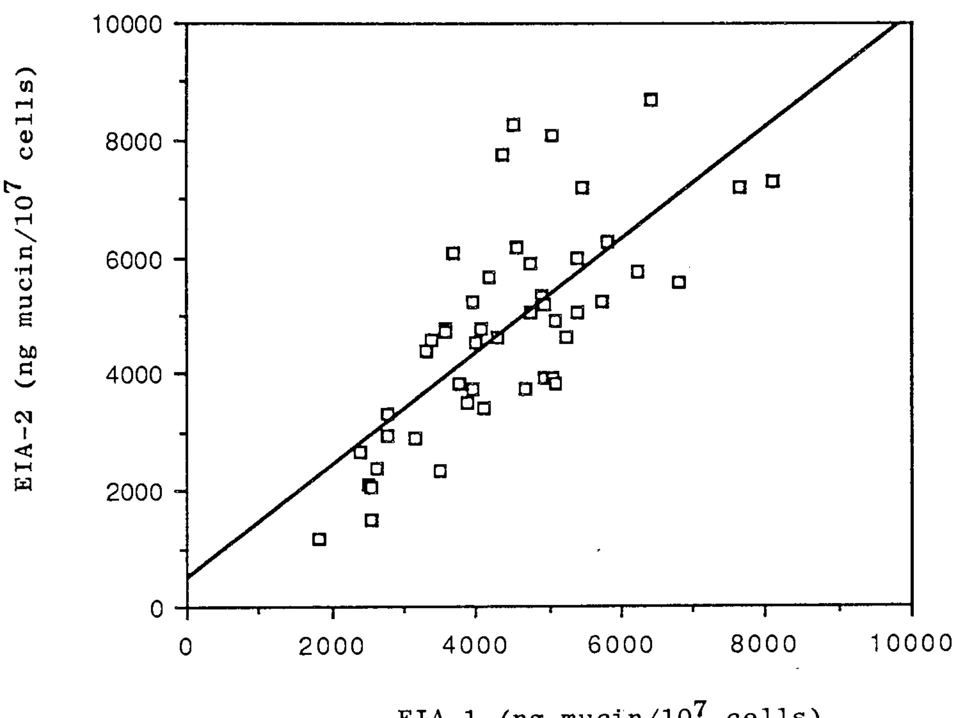
Over 15-60 min, release of mucin was essentially linear in the absence and presence of 50µM-forskolin (Fig 6.7B). At 45min stimulation with 50µM-forskolin represented a 48% increase over basal release, however at 60 min the stimulation had fallen to 12.5% of control. The reason for this fall in release may be related to the inherent variability that this assay system displayed (see 5.3.3.4). Alternatively accumulating proteolytic activity in the medium may be destroying the epitopes recognized by the antiserum, thereby underestimating the true mucin release. The affinity of the antibody preparation used in EIA-2 has been shown (Dekker et al., 1989b) to be lost upon incubation of 'reduced-denatured' mucin with proteinase K. The cellular content of immunologically detectable mucin, determined in a separate series of experiments was 72±12µg/10^7 cells (n=5).

6.3.1.3 Comparison of results obtained using EIA-1 and EIA-2

Visual inspection of the data presented in Fig 6.7 A,B, suggests that EIA-1 and EIA-2 recognize approximately equivalent amounts of immunoreactive material. However, results with EIA-2 are less satisfactory in that an effect of forskolin on the rate of mucin release (slope), rather than the amount released is not clearly demonstrable by contrast with EIA-1. Regression analysis (Fig 6.8) of the results obtained with the two assays, using 48 samples of incubation medium with mucin contents ranging from 74 to 324ng/40µl sample (assayed by 'native' assay) gave the following relationship:

Figure 6.8

Regression analysis of the relationship between EIA-1 and EIA-2.



The equation for the regression line is y=492.42 + 0.967x.

Result with EIA-2 = 19.7+ result with EIA-1 x 0.97

The correlation coefficient of 0.73 was significantly different from zero ($p<0.01$), the slope of the line (0.97) was not significantly different from one and the intercept (19.7) was not significantly different from zero (t-tests).

In conclusion, although the correlation between results obtained with EIA 1 and EIA 2 is not exact it would seem likely that both assays detect the secretion of rat gastric mucin from a suspension of isolated stomach cells. Both antibody preparations used in EIA-1 and EIA-2 have been fully characterized (5.3.1 & Dekker et al., 1989b) and shown to be specific for peptide epitopes present on rat gastric mucin. In addition both have been shown to exhibit a similar affinity for mucin secreted into incubation medium and their respective standard mucin (5.3.2.6 & 5.3.3.2). It is therefore not unreasonable that each assay should produce similar results given the similarities in their recognition of rat gastric mucin. Furthermore similiar results obtained with antibodies raised against different immunogens suggests that these assays are suitable for measuring mucin secretion although this material may not be of exactly the same structure as partially purified mucin scraped from the stomach surface.

In further experiments in this section only EIA-1 was used to estimate mucin secretion because of its lower variability (5.3.3.4) and clearer time-course result.

6.3.1.4 Discussion of [^{3}H]-labelled and bulk mucin time-course data

Results obtained with both the [^{3}H]-labelling protocol (4.3.2) and the solid phase EIA's appear to be the first direct measurements of [^{3}H]-labelled and bulk mucin release to have been obtained from an isolated

cell preparation. Both sets of data suggest that secretion of mucin is likely to be a of a slow continuous process moderately affected by secretagogues. Release of [^{14}C]-labelled high molecular mass glycoproteins from rabbit fundic mucosal explants pulse/chase labelled with [^{14}C]N-acetylglucosamine was found to be essentially linear over the 12 h experimental time course (Seidler et al., 1988). By contrast, direct measurements of the rat gastric surface mucus gel thickness after topical application of 10µg/ml 16.16-dimethyl prostaglandin E_2 showed that 70% of the maximum increase in gel thickness was obtained after 5 min (McQueen et al., 1983). It should be noted however that assessment of mucus gel thickness measures a dynamic steady state in which thickness will alter in the presence or absence of secretagogues, whilst the time-course data obtained in this work measures cumulative release (assuming no proteases in solution). Further possible reasons for the discrepancy between the direct measurement of gel thickness and [^{3}H]-labelled or bulk mucin release from isolated cells may be due to the former study utilizing an in vivo system, topical secretagogue application or a relatively high prostaglandin concentration (26µM).

Three mechanisms of release of canine gastric mucin have been observed (Zalewsky & Moody, 1979) in unstimulated mucosal biopsies: (i) slow release by exocytosis, (ii) rapid release by apical expulsion and (iii) cell exfoliation. Although ulcerogenic compounds such as aspirin which effect gross damage to the gastric mucosa may cause complete and rapid degranulation of mucous cells (Morris et al., 1984) the evidence from both sets of time-course data is that challenge with secretogogues does not produce this effect. The release of mucin in both cases was linearly related to time over the 15-60 min secretion period. Furthermore the low release of mucin at 60 mins expressed as a percentage of cellular material in

each case is compatible with a slow secretion by exocytosis over this period rather than a major burst of mucin release.

6.3.2 Effects of concentration of PMA, forskolin and A23187 on immunologicaly detectable mucin release

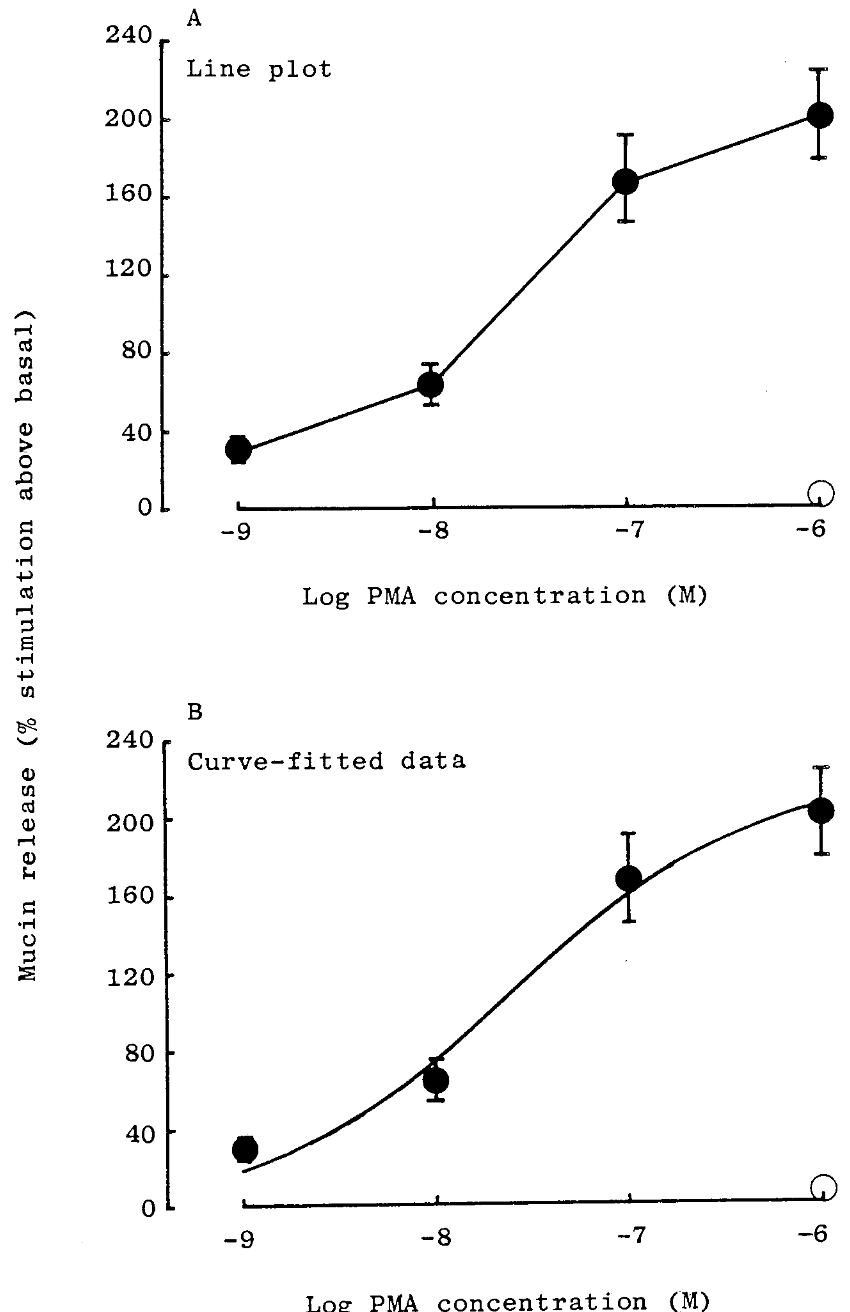
6.3.2.1 Effect of 1-1000nM PMA

Increasing the PMA concentration from 1nM to 1000nM effected a dose-dependent increase in the release of mucin after a 60 min incubation (Fig 6.9). The near-maximally effective concentration of PMA (1µM) stimulated the release of mucin by 202.6±23.1% (n=3). In a separate series of experiments 1µM-PMA released 8.2±2.0% (n=5) of immunologically detectable cellular material. If cells were incubated with 1µM 4α-PMA, a phorbol ester which does not activate protein kinase C, an insignificant stimulation of 6.1±1.9% (n=3) was observed.

The half-maximally effective concentration (EC_{50}) of PMA as determined by FIT was 25nM. The EC_{50} values for effects of PMA on secretory activity seem to vary substantially with the preparation and molecule secreted. Thus, the EC_{50} for inhibition of histamine-stimulated acid secretion is 3nM (Hatt & Hanson, 1989) while that for stimulation of insulin secretion by rat insulinoma cells is approximately 100nM (Hutton et al., 1984). The concentration of PMA required to activate partially purified protein kinase C from bovine brain was 2.5nM (Arcoleo & Weinstein, 1985). However the effective concentration of PMA may be reduced in systems involving intact tissue by virtue of sequestration into cellular lipid. The above considerations coupled with the lack of effect of 4α-PMA suggest that the action of PMA found here was a specific one probably involving protein kinase C.

Figure 6.9

The effect of concentration of PMA on mucin secretion.



Results are presented as means ± S.E.M. from three batches of cells. (●) PMA, (○) 4α-PMA. Release in the absence of phorbol ester was 2.2 ± 0.15μg/10^7 cells.

PMA releases $[^{14}C]$-labelled material of high molecular weight from explants of rabbit gastric mucosa (Seidler & Sewing, 1989). However, the results obtained (approximately a 35% stimulation above basal release at 1μM-PMA) were much smaller than those obtained in this work, and the shape of the dose-response curve suggested that at 1μM-PMA the response was far from maximal. A possible explanation for the discrepancy between the present results and those of Seidler & Sewing (1989) may be that poor access of PMA to the cells and reduction of its effective concentration through non-specific tissue binding reduced its potency in the rabbit explant system. This highlights one of the major advantages of isolated cell preparations, namely the ability to accurately control the composition of the medium in direct contact with the cells.

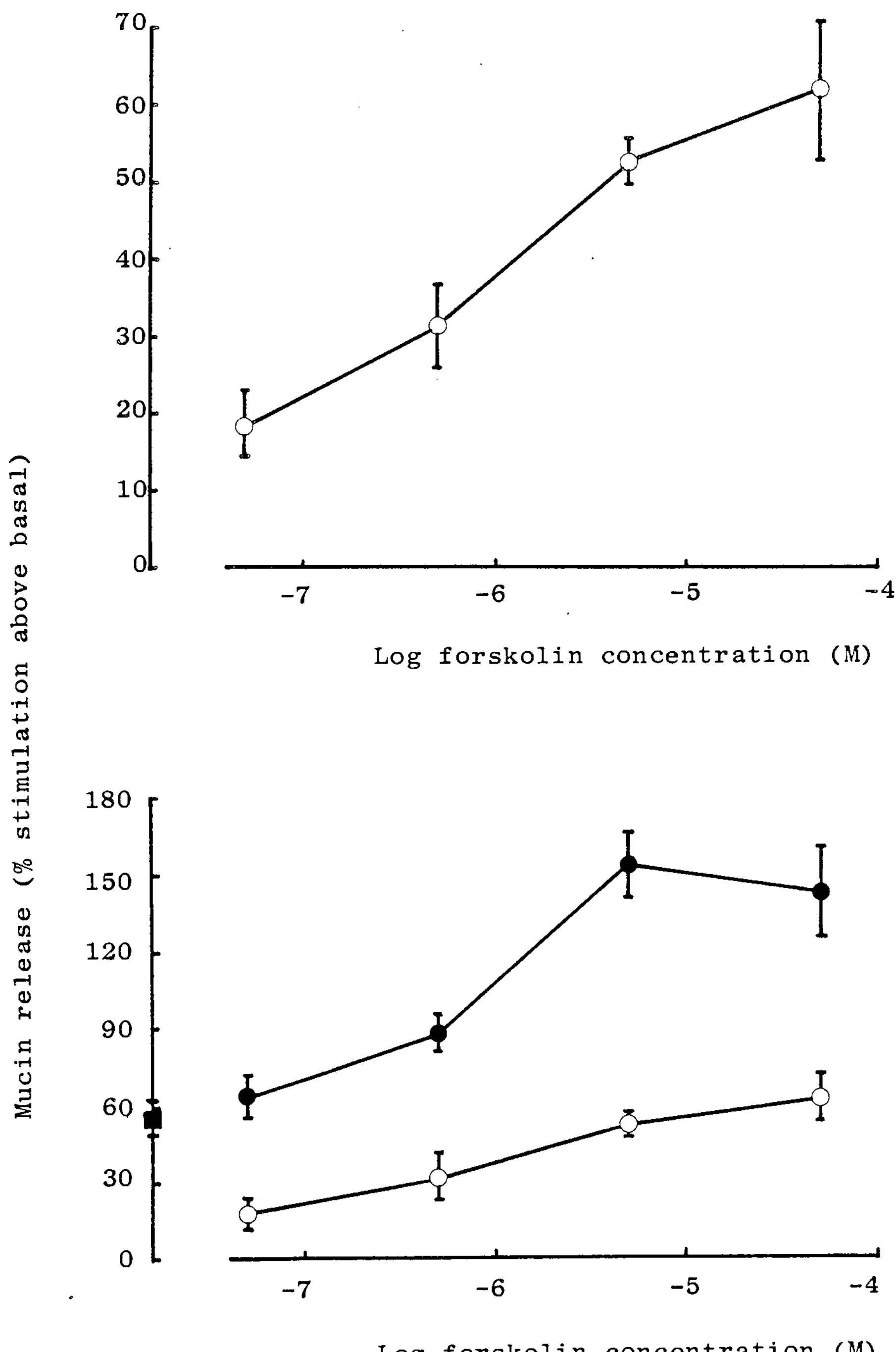
6.3.2.2 Effect of 0.05μM-50μM Forskolin

Forskolin stimulated secretion of mucin during a 60 min incubation. The release of mucin was found to be dependent on forskolin concentration over the range 0.05-50μM (Fig 6.10,A). Incubation with 50μM-forskolin gave a near-maximal response and stimulated the release of immunologically detectable mucin by 62.1±9.1% (mean ±SEM, n=4). The stimulation observed with 50μM-forskolin was significantly ($p<0.01$, t-test) lower than that observed with 1μM PMA (see 6.3.2.1).

The half-maximally effective concentration for forskolin required to elicit the stimulatory effect was 673nM. This result is higher than the EC_{50} for the stimulation of acid secretion from parietal cells (0.3nM; Chew, 1983) and lower than the EC_{50} reported for the stimulation of pepsinogen secretion from chief cells (3μM; Hersey *et al.*, 1983). The EC_{50} value for forskolin may vary because of differences between the cells in the basal state of G_s, because of differences

Figure 6.10

The effect of concentration of forskolin on mucin release and the interaction with 10nM-PMA.



Results are presented as means ± S.E.M. from four batches of cells. Incubation was for 1h in the presence of forskolin but the absence of PMA (○), in the presence of forskolin and 10nM-PMA (●) or with 10nM-PMA alone (■). Release of mucin in the absence of any additions was 2.0 ± 0.22μg/10^7 cells. The upper plot is presented to fully illustrate the effect of concentration of forskolin on mucin release.

in cyclic AMP phosphodiesterase activity between cells or because of a different sensitivity of secretory processes to changes in cyclic AMP concentration.

Seidler & Sewing (1989) using fundic explants of rabbit gastric mucosa found that 100μM-forskolin effected an 80% stimulation of the release of high molecular mass [^{14}C]-labelled material above basal levels and the EC_{50} was approximately 100nM.

6.3.2.3 Effect of 0.05μM-50μM forskolin in the presence of 10nM-PMA

Increasing the forskolin concentration from 0.05-50μM in the presence of 10nM-PMA caused a dose-dependent increase in the release of immunologically detectable mucin during a 60 min incubation (Fig 6.10, B). In the presence of 10nM-PMA the half maximally effective concentration of forskolin was approximately 1μM (visual estimation). At 5μM-forskolin there was a synergistic interaction between the forskolin and PMA. Thus the response expected from the addition of the effects of the two agents alone (108.2±7.5%; n=4) was significantly lower ($p<0.025$, t-test) than the result observed in the presence of both forskolin and PMA (153.1±13.5%; n=4).

Although the presence of a fixed concentration of PMA had little effect on the half-maximally effective concentration of forskolin, activation of protein kinase C by the phorbol ester enhanced the response to submaximal forskolin. Protein kinase C is probably activated by agents which induce receptor-mediated breakdown of phosphatidylinositol 4,5-bisphosphate and raise intracellular Ca^{2+} in mucous cells. This result implies that in the mucous epithelial cell there is a positive interaction (Nishizuka, 1986) between agents which activate cellular activity via effects on Ca^{2+}-dependent and cyclic AMP-dependant pathways.

In summary 10nM-PMA had little effect on the concentration of forskolin required for half-maximal response, but there was genuine synergism with 5µM-forskolin which was replaced by additivity of response at a higher concentration.

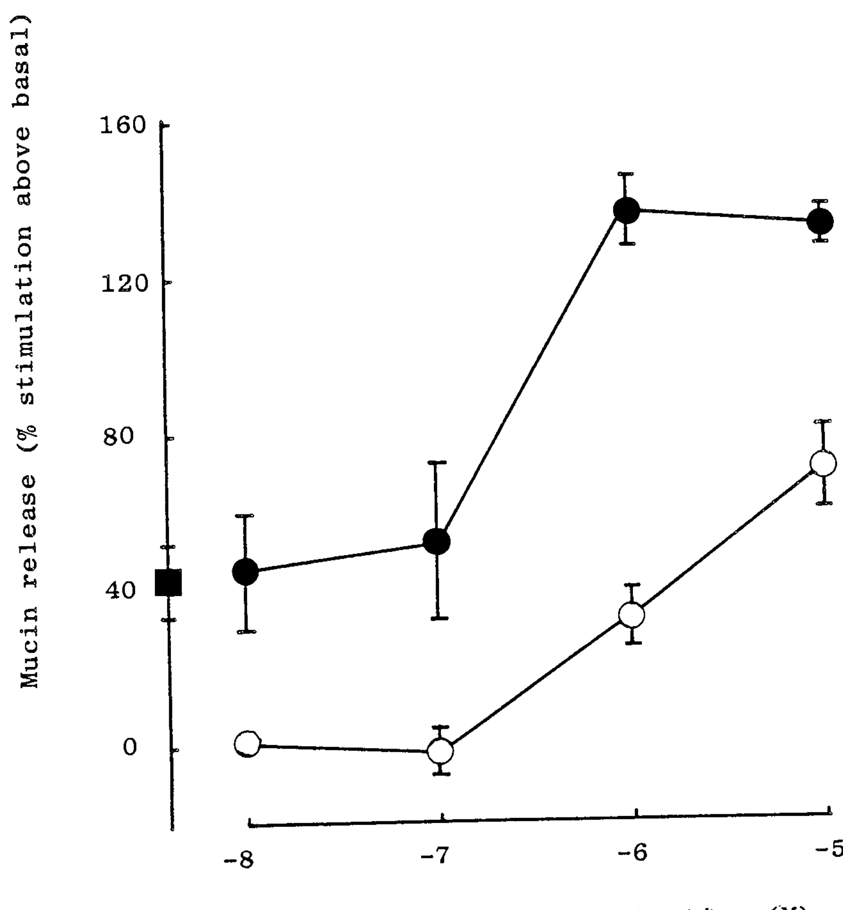
A number of points can be derived from this data. Firstly, elevation of cyclic AMP alone cannot produce a maximal secretory response. A single intracellular regulatory site (protein phosphorylation site) is therefore unlikely. Rather the data are compatible with two separate phosphorylation sites (site 1 involving protein kinase A and site 2 involving protein kinase C) on the same or different proteins. Site 2 phosphorylation is more effective at inducing secretion than site 1. However, submaximal phosphorylation of site 2 can influence the consequences of site 1 phosphorylation. Secondly, it seems unlikely that PMA elicits all of its effects on mucin secretion indirectly. The isolated cell preparation used in this work contains several cell types and PMA has been shown to increase endogenous prostaglandin E_2 levels in cultured rabbit gastric cells (Ota et al., 1990). Stimulation of rabbit mucin secretion via prostaglandins appears to be a cyclic AMP-dependent (Seidler & Sewing, 1989) process and therefore synergism/additivity with forskolin cannot be explained if PMA acts in this fashion.

6.3.2.4 Effect of 0.01µM-10µM A23187

Incubation of the isolated fundic cell suspension with the calcium ionophore A23187 stimulated immunologically detectable rat gastric mucin release. Secretion over a 60 min incubation period was found to be dose-dependent over the range 0.01-10µM (Fig 6.11; open circles). At 10µM A23187 mucin release was stimulated by 70.2±10.5% (n=3). The cytotoxicity of A23187 prevented the use of higher concentrations so no maximal response to this agent was obtained, nor could a half-maximally effective concentration value be

Figure 6.11

The effect of concentration of A23187 on mucin release and the interaction with 10nM-PMA.



Results are presented as means ± S.E.M. from three batches of cells. Incubation was for 1h in the presence of A23187 but the absence of PMA (○), in the presence of A23187 and 10nM-PMA (●) or with 10nM-PMA alone (■). Release of mucin in the absence of any additions was 2.2 ± 0.10μg/10^7 cells.

determined. A23187 (10μM) elicited a 50% stimulation of release of [^{14}C]-labelled high molecular mass material from fundic explants of rabbit gastric mucosa (Seidler & Sewing, 1989). Removal of extracellular calcium completely abolished this effect.

6.3.2.5 Effect of 0.01μM-10μM A23187 in the presence of 10nM-PMA

In the presence of 10nM-PMA, increasing the A23187 concentration from 0.01μM to 10μM dose-dependently stimulated the release of mucin during a 60 min incubation period (Fig 6.11; filled circles). The presence of 10nM-PMA can be seen by visual inspection to reduce the half-maximally effective concentration (EC_{50}) of A23187 to around 280nM. At 1μM A23187 there was a synergistic interaction between A23187 and PMA thus, the stimulation expected from the addition of the responses of the two agents alone (75.5±16.7; n=3) was significantly lower (p<0.025, t-test) than the effect obtained for the two agents together (134.6±9.4; n=3). This result is similar to that obtained for forskolin in the presence of PMA and suggests that a positive interaction exists between the activation of protein kinase C and the elevation of intracellular Ca^{2+}, the two signals probably generated by receptor-mediated polyphosphatidylinositol breakdown.

The demonstration that elevation of intracellular cyclic AMP (via forskolin; Seamon & Daly, 1987) or Ca^{2+} (via A23187; Pressman, 1976) and the activation of protein kinase C (via PMA; Nishizuka, 1986) can each independently initiate mucin secretion does not accord with the suggestion that the only role of mucin is to be rapidly released in response to epithelial damaging agents (Morris et al., 1984).

6.3.3 Effects of concentration of secretin and isoprenaline on immunologically detectable rat gastric mucin release

6.3.3.1 Effect of 0.1nM-100nM secretin

The release of mucin during a 60 min incubation was found to be stimulated by secretin in a dose-related manner ($p<0.01$ by analysis of variance; Fig 6.12). The half-maximally effective concentration was 3nM (manual estimation). The near maximal stimulation at 100nM was 34% above basal.

6.3.3.2 Effect of 10nM-10μM isoprenaline

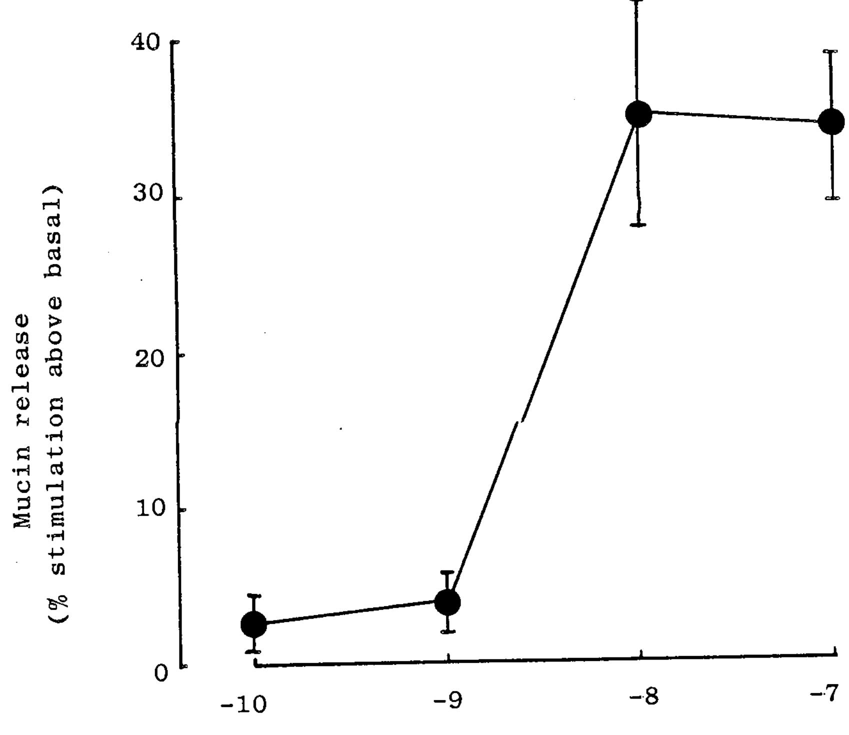
Release of mucin was also found to be related to the dose of isoprenaline ($p<0.01$ by analysis of variance; Fig 6.13). The stimulation at 10μM-isoprenaline was 19% above basal and the half-maximally effective concentration was 456nM.

6.3.3.3 Discussion of $[^3H]$-labelling and bulk mucin data for secretin and isoprenaline

Secretin and isoprenaline have previously been shown to release labelled Peak-1 material from isolated mucous epithelial cells in a dose-dependent manner. However it should be emphasized that these results could have reflected release of a small pool of mucin which had been labelled to a higher specific activity than the remainder. The solid-phase EIA system employed in this section of work measures bulk mucin secretion and probably therefore assesses the overall activity of the isolated mucous epithelial cells. The results presented in this section confirm the previous findings with the $[^3H]$-labelling method and suggest secretin and isoprenaline are important mucin secretogogues even when total secretory activity is assessed.

Figure 6.12

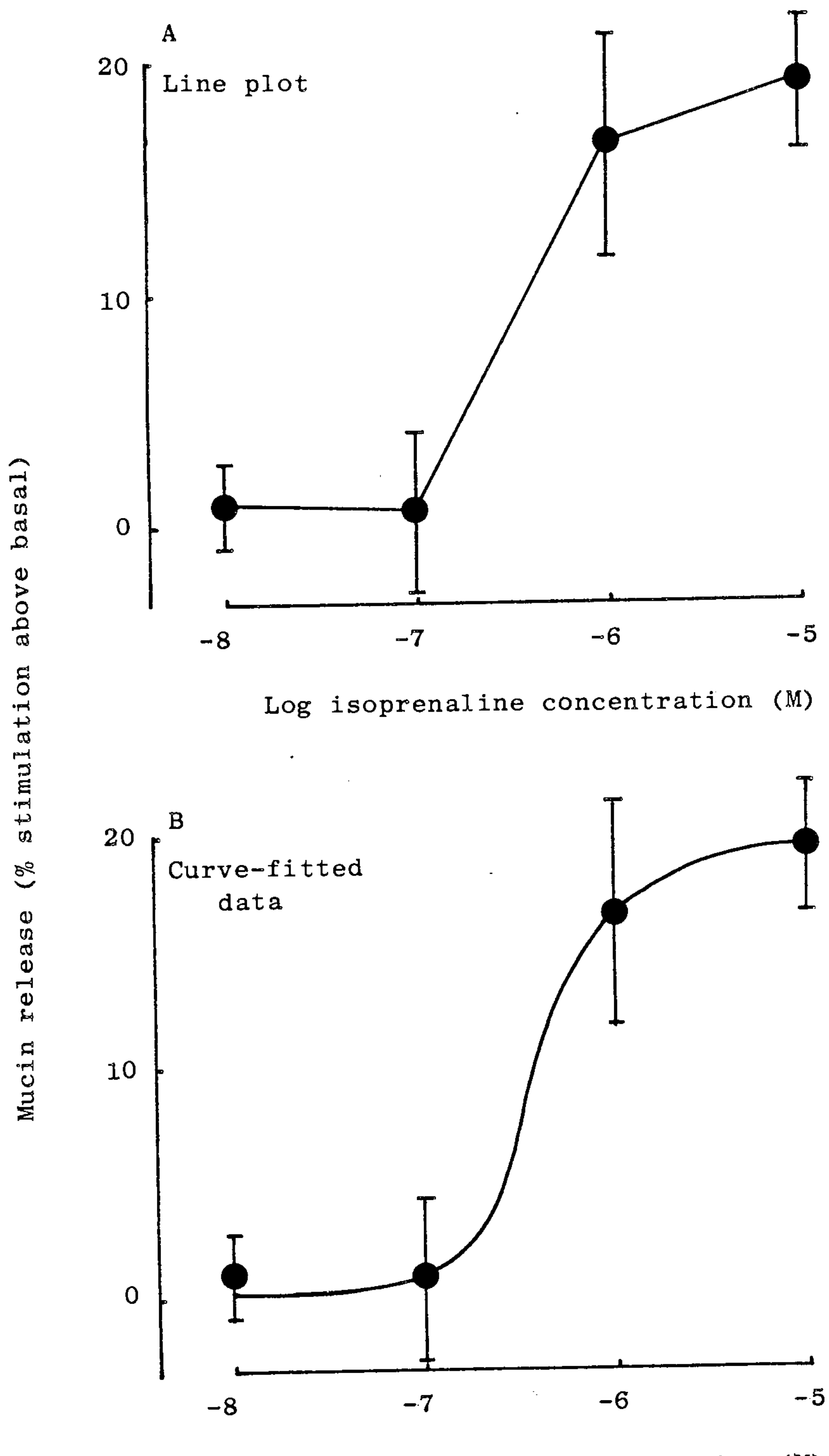
The effect of the concentration of secretin on mucin release.



Results are presented as means ± S.E.M. from four batches of cells. Control release was 2.1 ± 0.17 µg/10^7 cells. (This data could not be analysed by FIT because of the slightly lower value obtained at 10^{-7}M than 10^{-8}M secretin).

Figure 6.13

The effect of the concentration of isoprenaline on mucin release.



Results are presented as means ± S.E.M. from four batches of cells. Control release was 2.1 ± 0.07μg/10^7 cells.

There is strong evidence that secretin is a good mucin secretogogue (see Neutra & Forstner, 1987, for review). Secretin has been shown in cats to increase the content of sugars in the gastric juice although this finding could have been generated either from the degradation or release of mucin (Vagne & Perret, 1976). Intravenous infusion of secretin into rats in vivo increased the thickness of the mucous gel layer by approximately 75% over a 2h period (Allen et al., 1986). The relatively low EC_{50} concentration of secretin for stimulation of mucin release in both the [^{3}H]-labelling protocol and EIA-1 (approximately 3nM) suggests that this agent may be of physiological importance in mucin secretion. Secretin receptors coupled to adenylate cyclase have been detected in the rat gastric fundus (Gespach et al., 1980; Gespach et al., 1981) and it is possible that the effects of secretin are mediated by elevation of cyclic AMP levels in mucous epithelial cells.

A major novel finding of this work is that rat gastric mucin release can be influenced by adrenergic agents. Thus effects of adrenergic agents administered in vivo may be compromised by the indirect systemic effects of these agents in intact animals. The results obtained with both the [^{3}H]-labelling protocol and the solid-phase EIA illustrate one of the major advantages of isolated cell preparations, namely the ability to detect direct effects of potential secretogogues on specific cell types. Isoprenaline has been previously reported to stimulate submandibular mucin secretion in the rat (McPherson & Dormer, 1984). The intracellular signalling pathway used by isoprenaline remains to be established.

6.3.4 Effects of carbachol (100µM), IBMX (100µM) and TGLP-1 (100nM) on release of immunologically detectable rat gastric mucin.

The muscarinic cholinergic agonist carbachol (100µM) induced a small but significant (Table 6.1) release of immunologically detectable rat gastric mucin. Cholinergic stimulation using carbachol has been widely reported to greatly increase gastric mucous gel thickness in vivo (McQueen et al., 1983; Zalewsky et al., 1983). Release of high molecular mass [^{3}H]-labelled (Peak-1) material in response to 500µM-carbachol was significantly stimulated (18-29%) above basal levels, but this effect was small compared to the stimulation by 100nM-secretin (84%). The release of immunologically detectable gastric mucin by 100µM-carbachol in this work was similarly small compared to that released by 100nM-secretin. The reasons for this effect are unclear since the available evidence suggests carbachol should be a strong mucin secretogogue. A possible explanation is receptor damage during cell isolation e.g. proteolytic damage by pronase. A further possibility is that activation of the muscarinic cholinergic receptor by carbachol results in rapid feed-back inhibition of the receptor. It has been suggested that protein kinase C activated by carbachol may exert negative feedback inhibition of the muscarinic cholinergic receptor in parietal cells (Anderson & Hanson, 1984), possibly via receptor phosphorylation. Alternatively, only a small population of cells in the preparation may be responsive to carbachol stimulation.

100µM-IBMX stimulated mucin release (Table 6.1). The magnitude of this effect, which occurred in the absence of exogenous secretogogues, was somewhat surprising. Release of Peak-1 material was similarly increased (54.7%, n=2) above basal levels in the presence of 100µM-IBMX. IBMX is an inhibitor of cyclic AMP phosphodiesterase and an adenosine receptor antagonist. (Daley, 1982). Since forskolin is known

Table 6.1

Effect of carbachol (100μM), IBMX (100μM) and TGLP-1 (100nM) on the release of immunologically detectable rat gastric mucin

Agent	% stimulation above basal
Carbachol	8.9±1.2% (n=4)**
IBMX	43.2±10.2% (n=3)
TGLP-1	6.2±1.1% (n=3)*

The results are presented as means ±SEM with the number of cell-batches in parentheses. Release of mucin in the absence of any additions was 2.26±0.17μg/10^7 cells for carbachol data, 1.95±0.15μg/10^7 cells for TGLP-1 data and 1.87±0.18μg/10^7 cells for IBMX data.
* $p<0.05$; ** $p<0.01$ for the effect of incubation with agent by paired t-test.

to activate adenylate cyclase and has been demonstrated to stimulate the release of mucin, and IBMX is known to elevate intracellular cyclic AMP levels, it would seem likely that the effect of IBMX is mediated by increasing basal cyclic AMP levels. A further possibility is that endogenous activation of mucin release by prostaglandins and blockade of cyclic AMP phophodiesterase activity with IBMX is thus increasing the level of cyclic AMP and hence release of mucin. Evidence presented in 6.3.5 suggests that this scenario is unlikely. Alternatively, IBMX may antagonise the actions of endogenous adenosine binding to A_1 receptors. Binding of adenosine to A_1 receptors is known to reduce the activity of adenylate cyclase (Daly, 1985). Therefore elevation of cyclic AMP levels may result as a consequence of IMBX antagonism of A_1 receptors. However, there is no evidence to suggest that adenosine is an inhibitor of mucin secretion.

The very small stimulatory effect observed with TGLP-1 (Table 6.1) could be due to receptor damage during cell isolation. Alternatively the effects of TGLP-1 could be on another cell type. Firstly, the effects of secretin (an established mucin secretogogue) on cyclic AMP generation in rat fundic glands are additive to those of TGLP-1 (Hansen et al., 1988). Secondly, cyclic AMP generation induced by TGLP-1 and histamine in rat fundic cells is selectively inhibited by somatostatin, (Gespach et al., 1989) which strongly implies that TGLP-1 acts on parietal cells because secretin stimulation is not so inhibited and secretin receptors are present on chief and mucous cells. (Gespach et al., 1980; Gespach et al., 1981).

6.3.5 Effect of cyclo-oxygenase inhibition on release of rat gastric mucin.

Incubation of the isolated cell suspension with 10^{-5}M-indomethacin had little effect upon basal mucin secretion measured by EIA-1. Thus, release in the presence and absence of this agent over a 60 min

incubation was 1.093μg/ml cell suspension and 1.306 μg/ml cell suspension respectively (single experiment). In addition, little effect of 10^{-5}M-flurbiprofen on control release was observed when secretion was measured using the [^{3}H]-labelling/gel filtration protocol. In this system release in the presence and absence of flurbiprofen was 446 dpm/ml cell suspension and 440 dpm/ml cell suspension respectively (single experiment), after 45 min incubation.

The lack of effect of cyclo-oxygenase blockade in the isolated stomach cell preparation implies that endogenous prostaglandins do not affect basal mucin secretion. The prostaglandin E_2 concentration in the incubation medium of an enriched parietal cell fraction derived from Percoll density gradient centrifugation of the cell preparation used in this work was 56±11 pM (n=11) after 30 min incubation. (J.F. Hatt, PhD thesis, Aston University). The parietal cell enriched fraction has been demonstrated to produce more prostaglandin E_2 than the parietal cell depleted fraction of this gradient (J.F. Hatt, PhD thesis, Aston University) and it is unlikely therefore that prostaglandin E_2 levels in the 'crude' stomach cell preparation will exceed this concentration during a 30 min incubation. Furthermore since the half maximally effective concentration of prostaglandin E_2 required to induce secretion of [^{14}C]-labelled high molecular weight glycoprotein from rabbit mucosal explants was approximately 70nM (Seidler & Sewing, 1989), it seems probable that endogenous prostaglandin E_2 produced by the isolated cell suspension will be at an insufficient concentration to effect basal mucin secretion.

Incubation of rabbit mucosal explants with 10μM or 50μM-indomethacin significantly reduced the secretion of [^{14}C]-labelled high molecular mass glycoproteins over 16h incubation (Seidler et al., 1988). In contrast to the isolated stomach cell preparation the more intact explant system may facilitate the

production of high local concentrations of endogenous prostaglandins which could account for the observed effects of indomethacin in these systems.

In conclusion preliminary experiments investigating endogenous prostaglandin production suggest these agents do not have a major effects upon basal $[^3H]$-labelled or bulk mucin release, however further experiments are required to fully substantiate these findings.

6.4 SUMMARY

1) Effects of secretogogues on bulk mucin release in a suspension of isolated rat gastric cells can be detected using solid-phase enzyme immunoassay methodology.

2) Release of mucin in the presence or absence of 50µM-forskolin was essentially linearly related to time when assayed using EIA-1. Furthermore, after 60 min incubation stimulated cells released approximately 5% of the immunologically detectable cellular material, these results imply that secretogogues regulate a slow continuous mucin release.

3) PMA, forskolin and A23187 were found to stimulate bulk mucin release measured using EIA-1 in a dose-dependent manner. Synergistic interactions between PMA (10nM) and forskolin (5µM), and PMA (10nM) and A23187 (1µM) were observed.

4) Secretin and isoprenaline dose-dependently increased bulk mucin release measured using EIA-1.

5) A large stimulatory effect on bulk mucin release from the isolated cell preparation was demonstrated for 100µM-IBMX. Small but significant release of bulk mucin in response to 100µM-carbachol and 100nM-TGLP-1 was also observed.

Chapter Seven

GENERAL DISCUSSION

The major aims of this final chapter are (i) to compare results obtained with the [^{3}H]-labelling and enzyme immunoassay protocols and (ii) to compare the control of gastric mucin secretion with the control of mucin secretion in other regions of the gastrointestinal tract.

7.1 Comparison of results obtained with the [^{3}H]-labelling and enzyme immunoassay methodologies

Much of the previous work on the regulation of mucin secretion has used the technique of metabolically radiolabelling cellular mucin. The achievement of equilibrium labelling of the intracellular mucin pool is therefore of critical importance for accurately quantitating bulk mucin release in such experiments. In intestinal goblet cells, there is a subpopulation of mucin granules which are preferentially labelled and secreted, bypassing a pool of unlabelled granules (Neutra & Forstner, 1987). The possibility of different pools of mucin both within or between cells means that release of radiolabelled glycoprotein may not be an accurate measure of overall mucous cell activity. Enzyme immunoassay systems measure mucin release directly and therefore reflect bulk mucin release.

Examination of the time-course data obtained with the [^{3}H]-labelling protocol and the enzyme immunoassay (expressed as a % of equivalent cellular material) suggests that the former system gave results approximately two-fold greater than the latter. Thus release of Peak-1 material in the absence or presence of 1mM-dbcAMP and 0.5mM-carbachol was 5.7% and 10.4% over 60 min incubation, while release of immunologically detectable rat gastric mucin in the absence and presence of 50μM-forskolin was 2.6% and 4.7% respectively. The relatively low amount of cellular material released in both systems suggests

that secretogogues modulate basal exocytosis of mucin. Furthermore the similarity in the shape of the time-courses obtained with each system implies a common mechanism of release for Peak-1 and immunologically detectable material.

A comparison of the results obtained with near-maximal concentrations of secretin, isoprenaline and carbachol using the [^{3}H]-labelling and solid-phase enzyme immunoassay protocols is presented in Table 7.1. The stimulatory effect of these mucin secretogogues also appears to be at least two-fold greater using the radiochemical assay compared to the enzyme immunoassay. It should be noted that the data presented are not expressed equivalently since the [^{3}H]-labelling data is uncorrected for Peak-1 material present to t=0. Evidence (Fig 3.5) suggests that release of Peak-1 material at t=0 is low. The stimulatory effect of secretogogues using the radiochemical assay would be slightly greater if Peak-1 material at t=0 was taken into account.

The most likely explanation of the discrepancy observed between the two systems is that the isolated cell suspension used in this work contains more than one pool of mucin, the pool of mucin that is preferentially radiolabelled during a 2h incubation and 1h chase being more responsive to secretogogues. An alternative possibility is that the unavoidable absence of 0.5mM-dithiothreitol from the incubation medium in experiments using the enzyme immunoassay has led to an underestimation of secretogogue-stimulated mucin release in this system. 0.5mM-dithiothreitol has been shown (Table 4.3) to increase the release of Peak-1 material by approximately 38%, however it seems unlikely that the difference between the two assays can be explained by this effect alone because the

Table 7.1

A comparison of experimental results obtained with near-maximal doses of secretin, isoprenaline and carbachol+ using the [^{3}H]-labelling and enzyme immunoassay metholodologies.

	% stimulation of mucin in medium/release above basal*	
Agent	[^{3}H]-labelling	enzyme immunoassay
Secretin	84% at 100nM	34% at 100nM
Isoprenaline	34% at 10µM	19% at 10µM
Carbachol+	18% at 500µM	9% at 100µM

* Data obtained using the [^{3}H]-labelling protocol are % stimulation above basal mucin in medium since no value for mucin present at t=0 was subtracted. Data obtained using the enzyme immunoassay are corrected for mucin present at t=0 and are therefore % stimulation of mucin release during incubation. Lack of correction for labelled mucin at t=0 slightly underestimates the effect of secretogogues.

\+ Probably near-maximal by analogy with activation of muscarinic cholinergic receptors in other tissues.

stimulatory effect of carbachol using the radiochemical assay was not significantly affected by the presence or absence of dithiothreitol.

Labelled mucin release does not therefore provide a direct quantitative measure of total mucin release in vitro. In order to study the control of gastric mucin secretion accurately, it is desirable to measure bulk mucin. Furthermore the enzyme immunoassay as well as being capable of measuring more experimental samples in a shorter space of time, does not require purification of the sample before analysis and therefore represents a considerable advance upon the radiochemical assay.

7.2 Comparison of the control of gastric mucin secretion with other areas of the gastrointestinal tract

There is strong evidence that cholinergic innervation of the stomach and small/large intestine is important in the regulation of mucin secretion from these regions of the gastrointestinal tract. Secretion of gastric mucin in vivo in response to muscarinic cholinergic agonists such as acetylcholine or carbachol has been previously documented in this thesis (Table 1.4). In addition, carbachol has been demonstrated to induce a significant (although small) release of rat gastric mucin in vitro using methodologies developed in this work (Table 4.3, Table 6.1). Cholinergic stimulation of mucin release from small intestinal and colonic crypt (but not villus or surface) goblet cells has been demonstrated. Thus release of mucin in response to carbachol has been observed in rabbit duodenal, ileal and colonic organ cultures (Neutra et al., 1982), intact epithelial sheets of rat small intestine and colon (Phillips et al., 1984) and from a mucin-secreting clone (Cl.16E) derived from the HT-29 colonic epithelial cell line (Laburthe et al., 1989).

Activation of the secretory machinery using carbachol elevates the cytosolic level of Ca^{2+} and/or activates protein kinase C. Elevation of intracellular Ca^{2+} levels using the calcium ionophore A23187 or the activation of protein kinase C using the tumour promoter PMA have been shown to increase rat gastric mucin secretion in vitro from isolated stomach cells (6.3.2), rabbit gastric mucosal explants (Seidler & Sewing, 1989) and T84 human colonic adenocarcinoma cells (McCool et al., 1990). Furthermore the actions of these agents in the rat isolated mucosal cell preparation suggests a direct action of these compounds on the mucous cells in this system. These pathways could therefore mediate the effects of carbachol on mucin release in cells of the gastrointestinal tract.

In contrast to the findings with the muscarinic cholinergic agonists, agents which influence cyclic AMP metabolism have differential effects upon the secretion of mucin from the stomach, small intestine and colon. Thus elevation of intracellular cyclic AMP levels by forskolin or compounds such as secretin which probably raise cyclic AMP levels induced secretion of gastric mucin by the rat isolated mucosal cell suspension (6.3.2., 6.3.3). Similarly secretion of gastric mucin has been reported in response to forskolin and prostaglandin E_2 in rabbit mucosal explants (Seidler & Sewing, 1989). By contrast, incubation of rat intestinal loops (Roomi et al., 1984), rabbit duodenal, ileal and colonic organ culture (Neutra et al., 1982) or the CL.16E clone from human HT-29 colonic epithelial cell line (Laburthe et al., 1989) with dbcAMP or vasoactive intestinal peptide (VIP; a potent stimulator of intestinal water and electrolyte secretion) did not increase basal mucin secretion. However, stimulation of the CL.16E clone with VIP, dbcAMP or forskolin potentiated the stimulating effect of carbachol. This suggests that a functional adenylate cyclase signal transduction pathway exists in

these cells, but cyclic AMP per se is apparently unable to promote mucin secretion. Recently McCool et al., (1990) have demonstrated that VIP and prostaglandin E_1 both of which probably act through the adenylate cyclase pathway induce T84 human colonic adenocarcinoma cells to secrete mucin which was measured by solid-phase enzyme immunoassay. There is thus a suggestion that colonic mucus-secreting cell lines may respond to VIP but no such responses are found in those preparations of small intestine which have been investigated.

Exposure of the intestinal mucosa to cholera enterotoxin has been shown to result in a 4 to 10 fold increase in mucin secretion from rat small intestinal loops (Roomi et al., 1984) and the Cl.16E clone from HT-29 human colonic epithelial cells (Roumagnac & Laboisse 1989). Although the ADP-ribosylation of Gs by cholera toxin is well documented (Cassel & Sellinger, 1977) it would appear that its effects on intestinal mucin release are not cyclic AMP-dependent. Firstly, agents which raise intracellular cyclic AMP levels do not appear to induce small intestinal and colonic mucin secretion. Secondly, HT-29 18N2 cells (a goblet cell clone from HT-29 human colon carcinoma) do not show any degranulation as assessed by light microscopy upon incubation with cholera toxin (Lencer et al., 1990). These observations suggest that cholera toxin exerts its effects on small intestinal and colonic mucin secretion indirectly perhaps through mucosal nerves or other cell types.

Measurements of the secretion of bulk gastric and colonic mucin using solid-phase enzyme immunoassays suggest that secretogogues modulate a slow continuous secretory process. Thus activation of gastric mucin release with 50μM-forskolin induced the release of an extra 2.1% of cellular material over 60 min incubation (6.3.1.1). Similarly release of mucin from T84 human

colonic adenocarcinoma cells incubated with 1mM-carbachol (McCool et al., 1990) released 2% of total cellular mucin over a 30 min incubation.

In summary, secretion of gastric mucin can be promoted by the elevation of intracellular Ca^{2+}, cyclic AMP and the activation of protein kinase C. In contrast secretion of colonic mucin can be induced by activation of protein kinase C or elevation of intracellular Ca^{2+}, however elevation of cyclic AMP by itself is ineffective. Furthermore, secretion of gastric and colonic mucin are both likely to result from the modulation of a slow basal release.

PUBLICATIONS RESULTING FROM THIS WORK

Full Paper

A.C. Keates and P.J. Hanson (1990). Regulation of mucus secretion by cells isolated from the rat gastric mucosa. J. Physiol. 423, 397-409.

Communications

A.C. Keates and P.J. Hanson (1989). Characterization of labelled material secreted by a suspension of cells isolated from the rat gastric mucosa and preincubated with D-[6-^{3}H] glucosamine. Biochem. Soc. Trans. 17, 132-133.

A.C. Keates and P.J. Hanson (1990). Assay of mucus secreted from isolated stomach cells by using an ELISA. Biochem. Soc. Trans. 18, 956.

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APPENDICES

A1. Source of reagents

Reagent	Supplier
A. General Chemicals	
Agarose	Sigma
Ammonium sulphate	BDH
Basic Fuschin	BDH
Biotinylated donkey anti-rabbit immunoglobulin G	Amersham
Bovine serum albumin (BSA), fraction VI	ICN Biomedicals
5-Bromo-4-chloro-3-indoyl phosphate	Sigma
Caesium chloride	BDH
3-[(3-cholamidopropyl)-diamethyl ammonio]-1-propane sulphonate (CHAPS)	Sigma
Citric acid	BDH
DE52 cellulose anion exchanger	Whatman
De-fatted dried milk powder (Marvel)	Cadbury
Diaminobenzidine	Sigma
1,4-Diazabicyclo [2.2.2] octane (DABCO)	Sigma
Diethanolamine	BDH
Dimethylsulphoxide (DMSO)	Sigma
Disodium hydrogen orthophosphate	BDH
Disodium p-nitrophenyl phosphate	Sigma
Dithiothreitol (DTT)	Sigma
Ethylenediaminetetraacetic acid (EDTA)	BDH
'ExtrAvidin'	Sigma
Fluorescein isothiocyanate (FITC)-conjugated sheep anti-rabbit immunoglobulin G	Sigma
Foetal calf serum	Sigma
Freunds complete/incomplete adjuvant	Sigma
Gelatin	Sigma
Gentamicin sulphate	Sigma
L-glutamine	BDH
Glutaraldehyde	BDH
Glycerol	

Glycine	BDH
Hydrocortisone	Sigma
Hydrogen peroxide	BDH
N-2-hydroxyethylpiperazine-N'-2-ethane sulphonic acid (HEPES)	Sigma
Insulin	Sigma
Leupeptin	Sigma
Magnesium chloride, hexahydrate	BDH
Nitro blue tetrazolium	Sigma
Norit 'A' charcoal	Aldrich
Page blue 83	
Pepstatin A	Sigma
Periodic acid	BDH
Phenylmethylsulphonylfluoride (PMSF)	Sigma
Phosphotungstic acid	BDH
Potassium chloride	BDH
Potassium dihydrogen orthophosphate	BDH
Protein A - alkaline phosphatase conjugate	Sigma
Silver nitrate	BDH
Sodium azide	BDH
Sodium carbonate	BDH
Sodium chloride	BDH
Sodium dihydrogen orthophosphate	BDH
Sodium dodecyl sulphate (SDS)	BDH
Sodium hydrogen carbonate	BDH
Sodium hydroxide	BDH
Sodium metabisulphite	BDH
Sodium pentobarbitone (Sagatal)	May & Baker
Trichloroacetic acid (TCA)	BDH
Tris base	BDH
Trypan blue	BDH
Trypsin inhibitor, lyophillized from soybean	Sigma
Tween-20	Sigma

B. Enzymes

Chondroitinase ABC	Sigma
Hyaluronidase (type 1X)	Sigma
Papain	Sigma

Pronase	BDH

C. Radiochemicals and scintillation counting

'Econofluor'	New England Nuclear
[^{57}Co]cyanocobalamin	Amersham
D-[6-^{3}H]glucosamine hydrochloride	Amersham
'Optiphase Safe'	Pharmacia/LK
B	
'Protosol'	New England Nuclear

D. Secretogogues and agents

Calcium ionophore A23187	Sigma
Carbachol	Sigma
Dibutyryl cyclic AMP, sodium salt (dbcAMP)	Sigma
Epidermal growth factor (EGF)	Sigma
Flurbiprofen	Boots Co. Plc
Forskolin	Sigma
[Leu15]-gastrin (human)	Sigma
Histamine dihydrochloride	Sigma
Indomethacin	Sigma
3-isobuyl-1-methylxanthine (IBMX)	Sigma
(-)-Isoprenaline	Sigma
Phorbol-12-myristate-13-acetate (PMA)	Sigma
4 α-Phorbol-12-myristate-13-acetate	Scientific Marketing Associates
Secretin (porcine synthetic)	Sigma
Truncated glucagon-like peptide 1 (TGLP-1)	Peninsula Labs

A2 Preparation of Eagle's Minimum Essential Medium

The medium was purchased in powdered form from Sigma and contained the following components (final concentration):- L-Argenine (0.7mM), L-Cysteine (0.23mM), L-Glutamine (2.0mM), L-Histidine (0.27mM), L-Isoleucine (0.4mM), L-Leucine (0.4mM), L-Lysine (0.5mM), L-Methionine (0.09mM), L-Phenylalanine (0.2mM), L-Threonine (0.4mM), L-Tryptophan (0.05mM), L-Tyrosine (0.25mM), L-Valine (0.4mM), Choline chloride (7.0μM), Folic acid (3.0μM), Myo-inositol (0.01mM), Niacinamide (8.0μM), D-Pantothenic acid Ca (2.1μM), Pyridoxal HCl (4.86μM), Riboflavin (0.3μM), Thiamine HCl (3.0μM), $CaCl_2$ (1.8mM), KCl (5.4mM), $MgSO_4$ (0.8mM), NaCl (116.4mM), NaH_2PO_4 (1.0mM), D-glucose (5.6mM), Phenol Red Na (0.001% w/v).

The powdered medium was dissolved in 1l of distilled water with stirring at room temperature. To this $NaHCO_3$ (25mM) and HEPES (20mM) were added whilst stirring. This medium was warmed to 37^0C, gassed with 95% O_2/5% CO_2 for 30 min and the pH adjusted to 7.4.

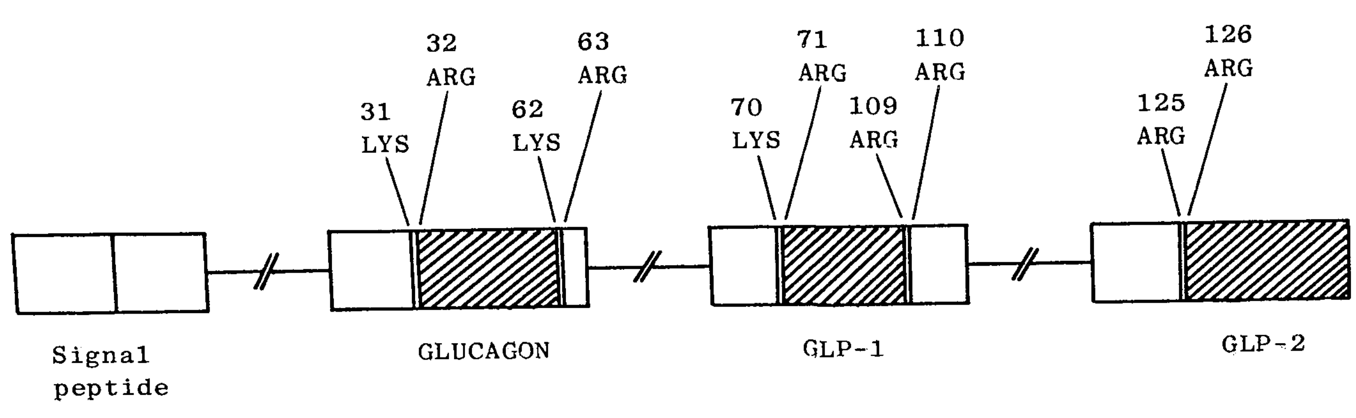
A3 Truncated Glucagon-like Peptide 1

The mammalian glucagon precursor (preproglucagon) is an 180 amino acid peptide (Bell et al., 1983; Fig A3.1). It contains pancreatic glucagon (preproglucagon 33-61), glicentin (preproglucagon 1-69), oxyntomodulin (pre-proglucagon 33-69). In addition it contains two glucagon-like peptides (GLP) designated GLP-1 (preproglucagon 72-108) and GLP-2 (preproglucagon 126-159) which have 50% homology with pancreatic glucagon. A truncated form of GLP-1 (TGLP-1; preproglucagon 78-108) has been isolated from porcine intestinal mucosa (Holst et al., 1987).

In man, pancreatic glucagon has been observed to stimulate fundic mucin secretion (Stachura et al., 1981). Recently, TGLP-1 has been reported to be a physiological ligand for glucagon-like receptors in the rat gastric mucosa and a potent agonist in the generation of cyclic AMP in rat fundic glands (Hanson et al., 1988). Its implication in mucin secretion has been suggested.

Figure A3.1

A schematic representation of the structure of Preproglucagon.



The amino acid residues highlighted represent cleavage sites between pairs of basic amino acids.

A4 Analysis of dose-response data

To increase the accuracy of determining the half-maximally effective concentration of an agent (EC_{50}) and the maximum response effected by that agent, dose response data were analysed by using the computer program FIT (Barlow 1983). This program fits experimental results by the method of least squares to the logistic expression.

$$Y = M \times \frac{X^P}{X^P + K^P}$$

Where:

X	=	concentration of agent	experimentally
Y	=	response	determined values
M	=	maximum response	
K	=	EC_{50}	
P	=	slope of linear part of curve relating X and Y.	

A graph of Y against log X is an S-shaped curve with the slope determined by P, and therefore represents a good model for a dose-response curve.

A5 Statistical Analysis

A5.1 Analysis of Variance (ANOVAR)

The analysis of variance used throughout this thesis was two-way, random block design. This has been applied (see below) to data from Fig 6.13 on the release of immunologically detectable rat gastric mucin in response to isoprenaline concentration (treatments), after a 45 min incubation. The mean squares are calculated by dividing the sum of squares by the corresponding degrees of freedom. The F-ratio is obtained by dividing the mean square for each factor by the residual mean square. The F-ratio is then compared with tabulated F-ratios with the degrees of freedom associated with the factor and residuals.

Variation	d.o.f.	Sum Sq.	Mean Sq.	F
Cell batch	3	289.23	96.41	3.11
Treatments	3	1175.94	391.98	12.63**
Residual	9	279.27	31.03	
Total	15	1744.44		

** $P<0.01$ with 3,9 degrees of freedom.

A6 Animals

Male Wistar rats, were obtained from Bantin and Kingman, Hull and were fed on Heygates breeding diet supplied by Pilsbury, Edgbaston, Birmingham.