http://researchspace.auckland.ac.nz

ResearchSpace@Auckland

Copyright Statement

The digital copy of this thesis is protected by the Copyright Act 1994 (New Zealand).

This thesis may be consulted by you, provided you comply with the provisions of the Act and the following conditions of use:

- Any use you make of these documents or images must be for research or private study purposes only, and you may not make them available to any other person.
- Authors control the copyright of their thesis. You will recognise the author's right to be identified as the author of this thesis, and due acknowledgement will be made to the author where appropriate.
- You will obtain the author's permission before publishing any material from their thesis.

To request permissions please use the Feedback form on our webpage. http://researchspace.auckland.ac.nz/feedback

General copyright and disclaimer

In addition to the above conditions, authors give their consent for the digital copy of their work to be used subject to the conditions specified on the Library Thesis Consent Form.
DEGRADATION AND UTILISATION OF MUCINS BY ANAEROBIC

BACTERIA FROM THE COLON

by

ROGER ANTHONY STANLEY

A thesis submitted to the University of Auckland for the degree of Doctor of Philosophy.

Department of Biochemistry

November 1982
To my parents for their encouragement and support of my education which has made this possible.
ACKNOWLEDGEMENTS

I would like to thank Dr A.M. Roberton for the opportunity to do the research for this thesis and for his guidance and supervision in seeing it to completion. I also gratefully acknowledge the other members of the Department of Biochemistry for their assistance and helpful discussions. Special thanks to Professor A.G.C. Renwick for his support during my years in the Department and to Drs. Sum Lee, Alison Wesley and members of the Department of Medicine for sharing their expertise in the field of mucin biochemistry.

Technical assistance has been expertly and graciously given by Mrs Joanna Wong. Thanks to Mrs Margery Smith and Mrs Margaret Smith for typing services and finally many thanks to Jan for her support and understanding.
ABSTRACT

Intestinal mucins are high-molecular-weight complex glycoproteins which are thought to be fermented by the anaerobic bacteria in the colon. The aim of this thesis was to investigate the mode and extent of mucin degradation by bacteria.

A method was developed to isolate gram quantities of soluble pig colonic mucin and pig gastric mucin. These preparations, along with commercially available pig gastric mucin, were incorporated into bacterial growth media as the sole carbohydrate energy sources. The molecular size and chemical composition of these mucins was determined before and after in vitro digestion by bacteria.

Three different studies were done. In the first, type cultures of Bacteroides fragilis were found to be capable of growing on gastric and colonic mucins by cleaving residues from the non-reducing ends of the mucin oligosaccharide chains. Mucin degradation, however, was limited to the removal of only a few residues although this may be significant in vivo as bacteria in the gut lumen must be able to utilise many different complex polysaccharides and thus do not specialise in degrading any one substrate.

In the second experiment, bacteria were isolated from the mucus layer of the pig colon. Three of the cultures studied extensively degraded mucins but colonic mucin was always more resistant to attack than the gastric mucins. Up to 80 mol% of the gastric mucin carbohydrates were cleaved and utilised. In contrast these bacteria utilised no more than 46 mol% of the colonic mucin carbohydrates.

In the third study, fresh rat faecal material was used to inoculate bacterial media containing either colonic mucin or commercial gastric
mucin. The faecal bacteria had completely degraded the gastric mucin after 24 h incubation but 36 h incubation was required to degrade the colonic mucin. However, when bacteria from the colonic mucin tubes were subcultured into fresh colonic mucin it was completely fermented after 24 h incubation.

It is hypothesised that, although the chemical compositions were similar, colonic mucin contained bonds that were not present in the gastric mucin. Evidence for this was obtained by assaying the mucins using the histochemical periodic acid/Schiff and Alcian blue stains. The Alcian blue reactivity was more conserved than PAS staining during bacterial hydrolysis. Therefore hydrolysis of these Alcian blue reactive groups may be the rate-limiting step in colonic mucin degradation.

The molecular basis of the PAS and Alcian blue stains was investigated. PAS stain (0.01% periodic acid) was found to react with terminal neuraminic acid, fucose and possibly galactose residues but not with hexosamine residues. Alcian blue stain bound mainly to sulphate esters but could not be correlated with total sulphate content. When both these colorimetric Alcian blue and PAS stains were used together they provided a valuable tool for assaying the results of chromatography runs. Gastric mucin was shown to be composed of separate neutral (Alcian blue negative) and acidic (Alcian blue positive) glycoproteins. Colonic mucin contained only acidic glycoproteins.
TABLE OF CONTENTS

Acknowledgements
Abstract
Table of contents
Abbreviations
Definitions

CHAPTER ONE

INTRODUCTION

GASTROINTESTINAL MUCUS GLYCOPROTEINS

1.1 Composition of mucus
1.2 Chemical composition of mucins
1.3 Physical structure of mucins
1.4 Subunit nature of some mucins
1.5 Sequences of the oligosaccharide side chains
1.6 Heterogeneity of mucins
1.7 Histochemistry of mucins and variation along the intestinal tract
1.8 Methods of extraction and purification of mucins from the gastrointestinal tract

DEGRADATION OF MUCINS

1.9 The mucus layer as a bacterial microenvironment
1.10 Physiological importance of bacteria-mucin interactions
1.11 Evidence that bacteria degrade mucins in vivo
1.12 Studies attempting to quantitate the numbers of mucin-degrading bacteria
1.13 Mucin degradation by bacteria from culture collections
CHAPTER TWO

MATERIALS

CULTURAL PROCEDURES

2.1 Maintenance and storage of bacterial cultures
2.2 Composition of the mucin-containing bacterial growth medium
2.3 Preparation and handling of agar media
2.4 Mucin sterilisation by filtration
2.5 Maintenance of anaerobic conditions outside the anaerobic chamber
2.6 Use of the anaerobic chamber

EXPERIMENTAL PROCEDURES

2.7 Gel filtration chromatography
2.8 Extraction of pig gastric and colonic mucins from fresh mucosal scrapings

ANALYTICAL METHODS

2.9 Colorimetric determination of hexose and fucose by the anthrone reaction
2.10 Determination of free neuraminic acid
2.11 Carbohydrate analysis by GLC
2.12 Periodic acid/Schiff colorimetric assay of glycoproteins
2.13 Colorimetric assay of glycoproteins using Alcian blue
2.14 Determination of sulphate content
# CHAPTER THREE

**MUCIN CHARACTERISATION**

<table>
<thead>
<tr>
<th>3.1</th>
<th>Ultracentrifugation of the mucins after PAS staining</th>
<th>51</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.2</td>
<td>Cellulose acetate electrophoresis</td>
<td>54</td>
</tr>
<tr>
<td>3.3</td>
<td>Digestion of the mucin preparations with hyaluronidase</td>
<td>58</td>
</tr>
<tr>
<td>3.4</td>
<td>Determination of the protein content by the Lowry and Coomassie blue protein assays</td>
<td>59</td>
</tr>
<tr>
<td>3.5</td>
<td>Effect of periodic acid concentration on the PAS reaction of mucins</td>
<td>63</td>
</tr>
<tr>
<td>3.6</td>
<td>Effect of pH on the Alcian blue binding to mucins</td>
<td>64</td>
</tr>
<tr>
<td>3.7</td>
<td>Effect of NaCl on Alcian blue binding to glycoproteins</td>
<td>67</td>
</tr>
<tr>
<td>3.8</td>
<td>Sepharose chromatography of the mucins</td>
<td>69</td>
</tr>
<tr>
<td>3.9</td>
<td>Effect of mild acid hydrolysis of the mucins on the PAS and Alcian blue reactions</td>
<td>72</td>
</tr>
<tr>
<td>3.10</td>
<td>Fractionation of the gastric mucin with CPC</td>
<td>79</td>
</tr>
<tr>
<td>3.11</td>
<td>Precipitation of mucins by acriflavine</td>
<td>82</td>
</tr>
<tr>
<td>3.12</td>
<td>Chemical composition of the mucins</td>
<td>84</td>
</tr>
<tr>
<td>3.13</td>
<td>Summary and conclusions</td>
<td>85</td>
</tr>
</tbody>
</table>

# CHAPTER FOUR

**UTILISATION OF MUCINS BY BACTERIA**

<table>
<thead>
<tr>
<th>4.1</th>
<th>Growth of <em>Bacteroides fragilis</em> on Sigma gastric and colonic mucin</th>
<th>87</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Growth of <em>B. fragilis</em> on the gastric mucin preparation</td>
<td>90</td>
</tr>
<tr>
<td>4.2</td>
<td>Degradation of mucins by isolates from the colonic mucosa</td>
<td>91</td>
</tr>
<tr>
<td></td>
<td>Isolation of the bacteria</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Identification of cultures</td>
<td>93</td>
</tr>
<tr>
<td></td>
<td>Utilisation of mucins by isolates from the pig colon</td>
<td>94</td>
</tr>
<tr>
<td>4.3</td>
<td>Degradation of mucins by rat faecal-pellet flora</td>
<td>99</td>
</tr>
</tbody>
</table>
CHAPTER FIVE

GENERAL DISCUSSION

5.1 Differences between the gastric and colonic mucin preparations

5.2 A possible mechanism for the failure of histochemical analysis to quantitatively detect mucin sulphate esters

5.3 Relationship between the moles of N-acetylgalactosamine and sulphate to the length of the mucin oligosaccharide chains

5.4 Resistance of colonic mucin to degradation

5.5 Difficulties in determining mucin degradation

5.6 Role of mucin degradation in the ecology of the gastrointestinal tract

5.7 Physiological implications of mucin breakdown

APPENDIX ONE

Bacterial contamination of Sigma gastric mucin

APPENDIX TWO

Mucinase assay of Wiel-Korstanje and Winkler (1975)

APPENDIX THREE

Labeling of mucins with Remazol brilliant blue

APPENDIX FOUR

Development of the colonic mucin extraction

APPENDIX FIVE

Calculation of sedimentation coefficients by method of Martin and Ames (1961)

REFERENCES
# LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Following</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1</td>
<td>Pig submaxillary glycoprotein</td>
</tr>
<tr>
<td>1.2</td>
<td>Sequences of rat colonic mucin oligosaccarides</td>
</tr>
<tr>
<td>1.3</td>
<td>Pig gastric glycoprotein</td>
</tr>
<tr>
<td>3.1</td>
<td>Density-gradient centrifugation of PAS labelled gastric and colonic mucins</td>
</tr>
<tr>
<td>3.2</td>
<td>Loss of Alcian blue reactivity from Sigma gastric mucin after digestion by hyaluronidase</td>
</tr>
<tr>
<td>3.4</td>
<td>Sigma gastric mucin in basal medium on Sepharose 4B</td>
</tr>
<tr>
<td>3.5</td>
<td>Modified Lowry protein assay of Miller and Hoskins (1981) on Sigma gastric mucin</td>
</tr>
<tr>
<td>3.6</td>
<td>Sepharose 4B of colonic mucin</td>
</tr>
<tr>
<td>3.7</td>
<td>Sigma gastric mucin on Sepharose 4B CL</td>
</tr>
<tr>
<td>3.8</td>
<td>Sigma gastric mucin on Sepharose 2B CL</td>
</tr>
<tr>
<td>3.9</td>
<td>Gastric mucin preparation on Sepharose 4B CL</td>
</tr>
<tr>
<td>3.10</td>
<td>Gastric mucin preparation on Sepharose 2B CL</td>
</tr>
<tr>
<td>3.11</td>
<td>Undegraded gastric mucin on Sepharose 2B CL</td>
</tr>
<tr>
<td>3.12</td>
<td>Effect of mild acid hydrolysis on the PAS and Alcian blue reactions of the mucins</td>
</tr>
<tr>
<td>3.13</td>
<td>Gastric mucin fractionated by CPC precipitation</td>
</tr>
<tr>
<td>4.1</td>
<td>Growth of <em>Bacteroides fragilis</em> on mucins</td>
</tr>
<tr>
<td>4.2</td>
<td>Sigma gastric mucin after digestion by <em>B. fragilis</em></td>
</tr>
<tr>
<td>4.3</td>
<td>Colonic mucin after digestion by <em>B. fragilis</em></td>
</tr>
<tr>
<td>4.4</td>
<td>Gastric mucin preparation after digestion by <em>B. fragilis</em></td>
</tr>
<tr>
<td>4.5</td>
<td>Growth on colonic mucin of serial dilutions of bacteria from pig colon mucus</td>
</tr>
<tr>
<td>4.6</td>
<td>Growth of cultures from the pig colon on mucins</td>
</tr>
<tr>
<td>4.7</td>
<td>Utilisation of Sigma gastric mucin by isolates from pig colon</td>
</tr>
<tr>
<td>4.8</td>
<td>Utilisation of the gastric mucin preparation by isolates from pig colon</td>
</tr>
<tr>
<td>Figure</td>
<td>Description</td>
</tr>
<tr>
<td>--------</td>
<td>-----------------------------------------------------------------------------</td>
</tr>
<tr>
<td>4.9</td>
<td>Utilisation of colonic mucin preparation by isolates from pig colon</td>
</tr>
<tr>
<td>4.10</td>
<td>Undigested Sigma gastric mucin on Sepharose 4B</td>
</tr>
<tr>
<td>4.11</td>
<td>Undigested gastric mucin preparation on Sepharose 4B</td>
</tr>
<tr>
<td>4.12</td>
<td>Undigested colonic mucin on Sepharose 4B</td>
</tr>
<tr>
<td>4.13</td>
<td>Sigma gastric mucin after digestion by culture 2</td>
</tr>
<tr>
<td>4.14</td>
<td>Gastric mucin preparation after digestion by culture 2</td>
</tr>
<tr>
<td>4.15</td>
<td>Colonic mucin after digestion by culture 2</td>
</tr>
<tr>
<td>4.16</td>
<td>Sigma gastric mucin after digestion by culture 13</td>
</tr>
<tr>
<td>4.17</td>
<td>Gastric mucin preparation after digestion by culture 13</td>
</tr>
<tr>
<td>4.18</td>
<td>Colonic mucin after digestion by culture 13</td>
</tr>
<tr>
<td>4.19</td>
<td>Sigma gastric mucin after digestion by culture 42</td>
</tr>
<tr>
<td>4.20</td>
<td>Gastric mucin preparation after digestion by culture 42</td>
</tr>
<tr>
<td>4.21</td>
<td>Colonic mucin after digestion by culture 42</td>
</tr>
<tr>
<td>4.22</td>
<td>Summary of Figures 4.10 to 4.21</td>
</tr>
<tr>
<td>4.24</td>
<td>Degradation of Sigma gastric mucin by rat faecal flora</td>
</tr>
<tr>
<td>4.25</td>
<td>Degradation of colonic mucin by complete rat faecal flora</td>
</tr>
<tr>
<td>Table</td>
<td>Following Page</td>
</tr>
<tr>
<td>-------</td>
<td>----------------</td>
</tr>
<tr>
<td>1.1</td>
<td>Composition of some gastrointestinal mucins 4</td>
</tr>
<tr>
<td>1.2</td>
<td>Gastrointestinal mucin molecular weights 7</td>
</tr>
<tr>
<td>2.1</td>
<td>Composition of the basal medium 30</td>
</tr>
<tr>
<td>3.2</td>
<td>PAS reactivity of sugars at 0.01% and 0.2% periodic acid 66</td>
</tr>
<tr>
<td>3.3</td>
<td>Effect of pH on Alcian blue binding 67</td>
</tr>
<tr>
<td>3.4</td>
<td>Effect of NaCl on Alcian blue binding 69</td>
</tr>
<tr>
<td>3.5</td>
<td>Analysis of mucin fractions 85</td>
</tr>
<tr>
<td>4.1</td>
<td>Mucin carbohydrates before and after digestion by <em>B. fragilis</em> 90</td>
</tr>
<tr>
<td>4.2</td>
<td>Properties of isolates from pig colon 95</td>
</tr>
<tr>
<td>4.3</td>
<td>Utilisation of Sigma gastric mucin by isolates from pig colon 97</td>
</tr>
<tr>
<td>4.4</td>
<td>Utilisation of the gastric mucin preparation by isolates from the pig colon 97</td>
</tr>
<tr>
<td>4.5</td>
<td>Utilisation of colonic mucin by isolates from the pig colon 97</td>
</tr>
<tr>
<td>4.6</td>
<td>Growth of rat faecal flora on mucins 102</td>
</tr>
<tr>
<td>4.7</td>
<td>Utilisation of anthrone-reactive sugars of colonic mucin by rat faecal flora 102</td>
</tr>
<tr>
<td>6.1</td>
<td>Mucinase test of Wiel-Korstanje and Winkler (1975) 130</td>
</tr>
<tr>
<td>6.2</td>
<td>Effect of pH on the mucinase assay of Wiel-Korstanje and Winkler (1975) 130</td>
</tr>
<tr>
<td>6.3</td>
<td>Mucin sedimentation coefficients by method of Martin and Ames (1961) 147</td>
</tr>
</tbody>
</table>
ABBREVIATIONS

Carbohydrates

Fuc         Fucose
Gal         Galactose
GalNAc      N-Acetylgalactosamine
Glc         Glucose
GlcNAc      N-Acetylglucosamine
Man         Mannose
NeuNAc      N-Acetyleneuraminic acid
NeuNGc      N-Glycolylneuraminic acid

General

BSA         Bovine serum albumen
CEC         Critical electrolyte concentration
CPC         Cetylpyridinium chloride
DMCC        Direct microscopic clump count
EDTA        Ethyldiaminetetraacetic acid
GLC         Gas-liquid chromatography
PAS         Periodic acid/Schiff (stain)
SDS         Sodium dodecyl sulphate
VFA         Volatile fatty acid
DEFINITIONS

The biochemical and histochemical nomenclature of mucus is often ambiguous. It has been recently reviewed by Reid and Clamp (1978) and the following terms have been used in this thesis:

Acidic and neutral mucins: These are used in a histochemical context and denote the staining reaction of the mucins.

Glycoprotein: Protein possessing covalently attached sugars.

Glycosaminoglycan: Carbohydrate portion of connective tissue proteoglycans.

Mucin: Principal glycoprotein component of mucus.

Mucus: Total secretion of the mucous membrane.

Sol/gel: The separation resulting from low-speed centrifugation of mucus to give a soluble sol phase and a precipitated gel phase.
CHAPTER ONE

INTRODUCTION

Studies on the nutrition and metabolism of colon anaerobes have greatly increased since the development of techniques to grow many of the $10^{12}$ bacteria per gram (dry weight) found in the faeces. Investigators have aimed at determining the physiological importance of these bacteria to the host. Around 15g dry wt of bacterial cells are excreted daily by an adult man consuming a typical western diet. It has been estimated that to produce this quantity of bacterial biomass requires the fermentation of 60 grams of carbohydrate (Cummings, 1981). The amount of dietary carbohydrate which passes into the colon each day is around 20g dry wt. Thus, to sustain the known bacterial growth three possibilities exist. The first is that more carbohydrate passes into the colon each day than is currently believed. The second is that the yield of bacterial cells per gram of carbohydrate is higher in the colon than in the rumen, which is the model on which the most information is available. The third is that material from the gut and lungs makes up the difference (Cummings, 1981). This last possibility would include mucus secretions from the lungs, mouth, stomach and intestinal canal as well as sloughed epithelial cells, their cell surface glycoproteins and connective tissue (Vercellotti et al., 1977). It is the nutritional potential of mucus in this latter category which is the main topic investigated in this thesis.

A dual approach for the investigation was adopted. In order to obtain the mucin substrates for in vitro growth studies on bacteria, a knowledge of the biochemical and physical properties of mucins was necessary. Methods for the extraction of gram quantities of mucins and their
characterisation were developed. When these mucin substrates became available they were used, together with anaerobic microbiological techniques, to isolate mucin-degrading bacteria. Pure cultures of these bacteria were studied to determine the mode and extent of in vitro mucin breakdown. Accordingly the introduction to this thesis has been divided into two main parts:

(1) Studies on the composition, function and properties of gastrointestinal mucins.

(2) Investigations by other workers into the bacterial breakdown of growth on mucins.
1.1 Composition of mucus

Investigations into the structure and function of mucus have been inspired by speculation about the possible roles it may have in the gastrointestinal tract. These proposed roles have included lubrication, mucosal surface protection, waterproofing and bacterial interaction (Forstner, 1978). Abnormalities in mucus composition or secretion have been implicated in pathological states such as gastric and duodenal ulceration, ulcerative colitis, gallstone formation and cystic fibrosis (Forstner, 1978; Lee et al., 1979). Evidence for any of these roles has been largely inferential and this field is still one of active research.

Techniques have been developed to separate and characterise the constituents of mucus in as undegraded state as possible (Creeth and Denborough, 1970; Snary and Allen, 1971). The high-molecular-weight (> 2 x 10^6) glycoprotein component, termed mucin, appears to be responsible for the rheological properties of mucus (Allen and Snary, 1972; Allen, 1981). In some cases other components such as DNA, (Ferencz, 1980) or serum proteins (List et al., 1978) may contribute to the viscosity of mucin solutions.

1.2 Chemical composition of mucins

The composition of gastrointestinal mucins has been extensively reviewed in recent years (Gottschalk, 1977; Horowitz, 1977; Allen, 1981). A representative table is reproduced for illustrative purposes (Table 1.1). Up to five different monosaccarides are found in all the mucin glycoproteins so far reported. These are
<table>
<thead>
<tr>
<th>Type</th>
<th>Degradative Treatment</th>
<th>Molar Ratios</th>
<th>% by Weight</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Fuc</td>
<td>Gal</td>
</tr>
<tr>
<td>1 Pig gastric</td>
<td>None</td>
<td>1.9</td>
<td>2.9</td>
</tr>
<tr>
<td>2 Human gastric</td>
<td></td>
<td>2.1</td>
<td>3.1</td>
</tr>
<tr>
<td>3 Pig intestinal</td>
<td>None</td>
<td>0.26</td>
<td>0.64</td>
</tr>
<tr>
<td>4 Human intestinal</td>
<td>None</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5 Pig Colonic</td>
<td>Pronase</td>
<td>1.5</td>
<td>2.5</td>
</tr>
<tr>
<td>6 Pig Colonic</td>
<td>Trypsin &amp; Pronase</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7 Human Colonic</td>
<td>None</td>
<td>0.46</td>
<td>2.7</td>
</tr>
<tr>
<td>8 Rat Colonic</td>
<td>None</td>
<td>4.7</td>
<td>1.0</td>
</tr>
</tbody>
</table>

- Terminal GalNAc residues / Internal GalNAc residues
- N-acetylgalactosamine / N-glycolylneuraminic acid

References:
1. Allen & Snary, 1972
2. Schrager & Oates, 1971
4. Jabbal et al., 1976
6. Inoue et al., 1966
7. Gold et al., 1981
8. Murty et al., 1978
fucose, galactose, N-acetylglucosamine, N-acetylgalactosamine and sialic acid. The sialic acids are either N-acetyl- or N-glycolyl-
neuraminic acids and they may also be O-acetylated in a variety of positions (Reid et al., 1975). Trace quantities of mannose and glucose have often been reported for purified gastrointestinal mucins although they may be due to bound, serum-type glycoproteins (Clamp et al., 1979). Uronic acids are never found in mucins, a feature that distinguishes them from the acidic glycosaminoglycans such as chondroitin sulphate and hyaluronic acid. These compounds, however, are sometimes isolated from the gastrointestinal tract along with mucins (Pamer et al., 1968). Ester sulphate is also found in some mucins (see Table 1.1) and was linked as N-acetyl-
glucosamine-6-sulphate in the one example in which the structure was determined (Slomiany and Meyer, 1972) although hexosamine disulphated sugars, as well as galactose sulphate, are also known to occur (Liau and Horowitz, 1982).

The protein content of mucins is usually less than 30% (w/w) of the composition and the relative abundance of amino acids is very similar for all mucins from the gastrointestinal tract (Forstner, 1978). Serine, threonine and proline are found in high concentration and together can exceed 50 mol% of the amino acid content (Allen, 1978).

1.3 Physical structure of mucins

A model for the structure of mucins has arisen from chemical and physical studies of their properties. The carbohydrate chains of mucins are linked to a central protein core forming a "bottle
brush" type of molecule. The linkage for epithelial mucins is always an alkali labile, O-glycosidic bond between N-acetyl-galactosamine and serine or threonine (Zinn et al., 1977). On average every third or fourth amino acid residue is a serine or threonine and all can be glycosylated. Peptides containing up to five adjacent serine or threonine residues have been sequenced indicating that very close packing of the carbohydrate chains occurs (Goodwin and Watkins, 1974). This tight packing is thought to provide a protective sheath of oligosaccharides along the protein backbone preventing even non-specific proteolytic enzymes from cleaving the molecule (Allen, 1981).

1.4 Subunit nature of some mucins

Techniques for isolating and characterising mucins in the undegraded form have been developed over the last decade primarily by the groups of A. Allen at the University of Newcastle (Allen and Starkey, 1974; Snary and Allen, 1972; Pearson et al., 1981; Marshall and Allen, 1978) and G. Forstner (Qureshi et al., 1979; Forstner et al., 1973) at the University of Toronto. They initially studied animal models because the material was more readily available and have since applied the methods to the study of human mucins.

Allen and co-workers used the pig as the experimental animal because the gastroenterology was similar to that of man (Montagna, 1959). High-molecular-weight pig gastric mucin was isolated without proteolytic degradation and was found to be cleaved into subunits by either disulphide bond cleavage or proteolysis. These studies have been extended to investigations of the pig small intestinal
mucus (Mantle and Allen, 1981), pig colonic mucus (Marshall and Allen, 1978) and human gastric mucin (Pearson et al., 1980). In all cases the native molecule can be degraded into constituent subunits. A protein, specifically released by disulphide bond cleavage, has been found in small intestinal and gastric mucins (Pearson et al., 1981). This protein has been implicated as an interlinking protein between the subunits. The results of these studies are summarised in Table 1.2.

Not all mucins have been shown to consist of a polymeric structure. Rat intestinal mucin (Forstner et al., 1973) and sheep submaxillary mucin glycoproteins (Holden et al., 1971) have been reported as not forming subunits with either disulphide bond cleavage or proteolysis treatment, but subsequent work has challenged this data. Rat intestinal mucin has been found to form subunits and release a peptide under very strong reducing conditions (A. Wesley, Personal communication).

1.5 Sequences of the oligosaccharide side chains

Although the sugar residues of the carbohydrate chains are limited to different combinations of the five so far documented (see Table 1.1), the number of residues per chain and the relative amounts of each sugar are very variable among the different mucins. The development of techniques to isolate and sequence chains has led to several glycoproteins being more fully characterised. Reduced oligosaccharides are obtained by cleavage of the alkali labile carbohydrate-protein bonds followed by borohydride reduction.
<table>
<thead>
<tr>
<th>Type</th>
<th>Ref.</th>
<th>Undegraded molecule</th>
<th>Smallest subunit</th>
<th>Interlinking peptide</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pig gastric</td>
<td>a</td>
<td>$2 \times 10^6$</td>
<td>$0.50 \times 10^6$</td>
<td>70,000</td>
<td>e</td>
</tr>
<tr>
<td>Pig intestinal</td>
<td>b</td>
<td>$1.7 \times 10^6$</td>
<td>$0.45 \times 10^6$</td>
<td>90,000</td>
<td>f</td>
</tr>
<tr>
<td>Pig colonic</td>
<td>c</td>
<td>$15 \times 10^6$</td>
<td>$0.76 \times 10^6$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Human gastric</td>
<td>d</td>
<td>$2 \times 10^6$</td>
<td>$0.53 \times 10^6$</td>
<td>70,000</td>
<td>e</td>
</tr>
<tr>
<td>Rat intestinal</td>
<td>g</td>
<td>$2 \times 10^6$</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

a. Starkey et al., 1974
b. Mantle & Allen, 1981
d. Pearson et al., 1980
e. Pearson et al., 1981
f. Mantle, Mantle & Allen, 1981
g. Forstner, Jabbar et al., 1973
The oligosaccharides are separated by chromatography and the sequence determined by combinations of compositional analyses, immunological assay, specific glycosidase action and methylation studies (Slomiany et al., 1980).

Published sequences of some relevant mucins have been reproduced in Figures 1.1 and 1.2. In pig submaxillary mucin (Fig. 1.1) there are up to five sugar residues per chain but the full pentasaccharide formed only 9% of the total number of sugar chains. The major fraction was a single N-acetylgalactosamine residue.

The carbohydrate sequences of rat colonic glycoprotein (Fig. 1.2) also show that many variations of the maximum chain exist. In this case up to twelve sugar residues per chain were determined by sequencing (Slomiany et al., 1980) but this glycoprotein was reported to have an average of 22 residues per oligosaccharide chain (Murty et al., 1978). This was determined by measuring the number of alditol residues formed from the linking galactosamine sugar by alkali elimination and borohydride reduction of the whole glycoprotein. Thus the average chain length was the total number of moles of sugar divided by the number of moles of linking N-acetylgalactosamine. Human colon mucin samples have also been analysed by this method (Clamp et al., 1981). A broad spectrum of oligosaccharide chain lengths was found. The number of residues per chain varied from a minimum of two to greater than 15.

Several features of the oligosaccharide chains can be noted. Fucose is always linked at a non-reducing terminal. The glycosidic
Figure 1.1  Pig submaxillary glycoprotein

<table>
<thead>
<tr>
<th></th>
<th>Structure</th>
<th>% of total number of oligosaccharides</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>GalNAc-ol</td>
<td>35%</td>
</tr>
<tr>
<td>2</td>
<td>Gal 1→3GalNAc-ol</td>
<td>2%</td>
</tr>
<tr>
<td>3</td>
<td>NeuNGc2→6GalNAc-ol</td>
<td>12%</td>
</tr>
<tr>
<td>4</td>
<td>Fuc1→2Gal 1→3GalNAc-ol</td>
<td>11%</td>
</tr>
<tr>
<td>5</td>
<td>NeuNGc2→6Gal 1→3GalNAc-ol</td>
<td>15%</td>
</tr>
<tr>
<td>6</td>
<td>Fuc1→2Gal 1→3GalNAc-ol</td>
<td>5%</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>GalNAc1→3Gal 1→3GalNAc-ol</td>
<td>9%</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>GalNAc1→3Gal 1→3GalNAc-ol</td>
<td>11%</td>
</tr>
</tbody>
</table>

References:

a. Calson, (1968)

b. This structure has been refuted by Haleek et al., (1981)
Figure 1.2  Sequences of rat colonic mucin oligosaccharides

1. NeuAc2→6GalNAc-ol

2. Gal 1→4GlcNAc1→3GalNAc-ol

3. GlcNAc1→3GalNAc-ol
   6
   +
   2NeuAc

4. Gal 1→4GlcNAc1→3GalNAc-ol
   6
   +
   2 NeuAc

5. GalNAc1→3Gal 1→3/4GlcNAc1+3Gal 1→4GlcNAc1+3GalNAc-ol
   2
   6
   6
   +
   +
   1Fuc
   2NeuAc
   2NeuAc

6. GalNAc1→3Gal 1→3/4GlcNAc1
   2
   +
   1Fuc
   3/6Gal 1→4GlcNAc1→3GalNAc-ol
   6
   +
   NeuAc2→6Gal 1→4GlcNAc1
   2NeuAc

7. GalNAc1→3Gal 1→3/4GlcNAc1
   2
   +
   1Fuc
   3/6Gal 1→4GlcNAc1→3GalNAc-ol
   6
   +
   GalNAc1+3Gal 1→4GlcNAc1
   6
   +
   2NeuAc
   2NeuAc

Reference: Slomiany et al. (1981)
linkage of fucose in pig submaxillary and rat colonic mucin is by
an α1-2 bond to galactose. Human ovarian cyst mucin is proposed
to also have α1-3 and α1-4 fucose-N-acetylgalactosamine bonds (Lloyd

Neuraminic acid is also found in a non-reducing terminal
position. In rat colonic mucin and pig submaxillary mucin it is
linked to the reducing terminal N-acetylgalactosamine. Rat colonic
neuraminate is also linked to terminal galactose residues. Removal
of neuraminic acid by neuraminidase can increase the blood group
A activity of pig colon mucus (Marshall and Allen, 1978) but not
pig small intestinal mucus (Mantle and Allen, 1981). The increased
A-activity indicates neuraminic acid linkage at the non-reducing
terminal.

The position or positions of sulphate residues, if present,
run be difficult to determine. Slomiany and Meyer (1972), when
investigating pig gastric mucus, found that at least some of the
sulphate was esterified as N-acetylgalactosamine-6-sulphate (Fig.
1.3). This linkage also occurs in pig colonic mucin (Fukuda and
Matsumura, 1975) but esterification to vicinal hydroxyls in some
mucins has been proposed on the basis of histochemical data (Lev,
1977). The recent finding of disulphated hexosamines in gastric
mucin (Liau and Horowitz, 1982) strengthens this hypothesis. In
canine submaxillary mucus linkage is thought to be by a 3- or
4-sulphate ester to N-acetylgalactosamine (Lombart and Winzler, 1974).

1.6 Heterogeneity of mucins

The concepts of homogeneity and heterogeneity that are
Figure 1.3  Pig gastric glycoprotein

GalNAc1+3Gal 1→3/4GlcNAc1→4Gal 1
   2     2
  ↑     ↑
1Fuc   1Fuc

   6
  ↑
  SO₄

Fuc1→2Gal 1→3/4GlcNAc1→4Gal 1
   2
  ↑
  1Fuc

Reference: Slomiany and Meyer (1972)
applied to the analysis of proteins have been found not to be strictly applicable to the analysis of glycoproteins. Preparations that are free of extraneous impurities are often polydisperse with respect to charge, size and density when subjected to analyses such as ultracentrifugation or isoelectric focusing (Horowitz, 1977). The principal cause of heterogeneity is variations in the length and content of the carbohydrate chains. Figures 1.1 and 1.2 illustrate that mucin oligosaccarides occur in all the possible combinations of the maximum chain. This could be due to lysosomal or bacterial enzymatic degradation, incomplete synthesis by the glycosyl transferases or result from extraction from more than one cell type (Horowitz, 1977).

Microheterogeneity is a term used to represent variations resulting from glycosylations that do not have absolute specificity on the growing carbohydrate chain. This is most commonly associated with sialic acid or fucose where the mutual exclusion of these residues is found for any branch of a carbohydrate chain as illustrated by Figure 1.1. They may also act as chain terminators preventing the incorporation of other sugar residues (Beyer et al., 1979).

1.7 Histochemistry of mucins and variation along the intestinal tract

The histochemical characterisation of mucins in situ and the use of radioisotope-labelled precursors has been invaluable in characterising the production and variation of mucins along the gastrointestinal tract. Much of the interest has centred around the separation and identification of neutral glycoproteins from acidic
glycoproteins and whether they consist of the same structure except for the addition of charged groups (Horowitz, 1977).

The use of basic dyes at several pH values has been a useful way of selectively demonstrating acidic groups in glycoproteins. At pH 1.0 only sulphate groups will bind stain whereas at pH 2.5 both carboxyl and sulphate groups are ionised (Lev and Spicer, 1965). Mucosubstances with ester sulphate will also stain at higher ionic strength (> 0.2 M) than those with carboxyl groups (Scott, 1972). These properties are fundamental to the most commonly employed staining procedures using Alcian blue (Scott, 1972) and high iron diamine (Spicer, 1965) to visualise acidic mucins. The neutral mucins are readily detected by the periodic acid-Schiff (PAS) technique.

Normal gastric mucosa of the human stomach contains predominantly neutral mucins with only faint traces of acidic mucins in the mucus neck cells. Autoradiography and immunofluorescence, however, do demonstrate sulphated mucins in the gastric epithelium. While it is generally agreed that there is a predominance of neutral mucins in the gastric mucosa, the origins and proportion of the sulphated material is still a subject of debate (Filipe, 1979).

The small intestine exhibits a gradient of mucin types, both from the jejunum to the ileum and from the base of the crypts to the tip of the villi. This latter change is from neutral mucins produced by immature cells in the crypts to sialomucins being produced by the cells near the tip of the villi (Filipe, 1979).
and is paralleled by changes in the sialyl and glycosyl transferase activity (Isselbacher, 1974). The regional differences along the tract are a distally increasing content of O-acylated sialomucins with some sulphate esters near the terminal ileum. The sialomucins of the jejunum do not contain O-acyl neuraminic acids (Culling et al., 1977; Filipe, 1979).

As in the small intestine, the mucin composition of the colon varies both along its length, and from the base of the crypts to the tip of the villi. In the adult human left colon sulphomucins predominate in the lower half of the crypts while sialomucins and sulphomucins are seen in the upper half of the crypts. The more distal right colon has sialomucins present in the lower third of the crypts with a predominance of sulphomucins in the upper two thirds of the crypts (Filipe, 1979).

1.8 Methods of extraction and purification of mucins from the gastrointestinal tract

The classical method for glycoprotein extraction was developed by Morgan and King (1943) to extract pig gastric mucin. They employed autolysis, phenol extraction and precipitation with ethanol to obtain the blood group active glycoproteins. When later procedures were developed they often employed proteolytic enzymes instead of autolysis to solubilise the mucins from whole mucosa. All procedures have advantages and disadvantages and must be modified to suit the properties of the tissue being extracted.

The use of proteolytic enzymes facilitates the extraction
of what might otherwise be an insoluble gel. The effects of these enzymes was extensively investigated by Snary and Allen (1971) using radioactively-labelled mucins to follow the efficiency of extraction. They found that enzymes such as Pronase could release up to 93% of the radiolabel in the non-diffusible mucus material. When the water-soluble material was fractionated by Sepharose chromatography an excluded and an included peak were produced which were chemically and immunologically very similar (Snary and Allen, 1971). Thus it was shown that the enzymes released degraded glycopeptides. Attention was then focused on the development of non-degradative methods of extraction.

The use of equilibrium density centrifugation in CsCl (Creeth and Denborough, 1971) allowed the separation of the dense glycoproteins (p > 1.50g/ml) from the non-covalently bound protein (p < 1.450g/ml). Pig gastric mucin purified by this method could then be split into subunits by proteolytic enzymes or disulphide bond-cleaving reagents. However only a small proportion of the total glycoprotein could be extracted. Mucus gel is thought to be made insoluble by interactions between the mucoprotein molecules and between mucoproteins and other cellular material such as cationic proteins (Horowitz, 1978).

Marshall and Allen (1977) reported that the mucus glycoproteins from pig stomach mucosal scrapings could be made water soluble by dilution and homogenisation in a Waring blender. Subsequent purification was by repeated density centrifugation and/or repeated gel filtration. Up to four of these purification steps are necessary because at each step non-covalently bound protein
and nucleic acids are released from the main mucin fraction (Mantle and Allen, 1981).

The water solubilisation of the mucus gel can recover more than 80% of the mucin glycoprotein in pig stomach and small intestinal mucus (Mantle and Allen, 1981). Marshall and Allen (1978) reported that 72% of the freeze-dried weight of the pig colon mucosal scrapings was recovered in this way but only 5% of that weight was glycoprotein. The loss in the insoluble gel was not reported. Contaminating DNA and RNA was extracted along with the small intestinal and colonic mucus and was presumed to originate from the mucosal cells (Mantle and Allen, 1981; Mantle and Allen, 1978). The use of DNAase and RNAase was found to remove these materials (Marshall and Allen, 1978).

Glycoprotein composition can be a function of both the source of the material and the extraction method employed (Horowitz, 1977). If the carbohydrate chains have genetically determined antigenic specificities then the composition of the product will reflect whether samples from homozygote or heterozygote individuals were used (Horowitz, 1977). If solvent precipitation is employed, this method too can alter the final product composition. Glycoproteins with higher carbohydrate compositions will be precipitated out at lower solvent strengths. Likewise the use of cationic detergents such as cetyl pyridinium chloride or the use of anion exchange resins can affect the product by selecting those molecules with negative charges (Horowitz, 1977).
DEGRADATION OF MUCINS

1.9 The mucus layer as a bacterial microenvironment

The development of frozen-section histology (Savage et al., 1968; Davis, 1976) allowed the visualisation of bacteria in situ and revealed that the mucosal surface and associated mucus layer were colonised by a specific microbial flora. Oxygen intolerant anaerobic bacteria, spirochaetes and other spiral-shaped microbes colonise the caecal and colonic epithelium of rats, mice, dogs and other animals, including monkeys (Savage, 1972; Savage, 1975; Leach et al., 1973; Takeuchi et al., 1972). The stomach and small intestine of rodents have also been shown to harbour a specific epithelial flora (Savage, 1972; Savage, 1975). The epithelium and mucus layer constitute separate microbial habitats and the autochthonous bacteria found there appear highly adapted to their individual microenvironment.

The specificity of the different bacterial types for particular segments of the gut mucosa was demonstrated by Phillips et al. (1978). Factors governing the specificity of the bacteria for their region have yet to be elucidated but may include the type of mucin being secreted in that area, the type of villus cell, the rate of transit of luminal contents, the presence of unconjugated bile acids, volatile fatty acids and the washout rate of the secretions (Phillips et al., 1978; Savage, 1977). The ability to oppose the continuous secretions must be an important adaptation, particularly in the crypts, as most observed bacteria are either motile (e.g. the spirochaetes) or anchored (e.g. the filamentous
bacteria of the rat small intestine). If animals are purged with MgSO$_4$, large numbers of normally mucosal-associated bacteria are then found in the luminal contents (Phillips et al., 1978). Study of these mucosal-associated bacteria has been hampered because many have yet to be isolated and grown in recognisable form (Savage, 1977).

The nature of the luminal mucin layer, as opposed to mucin in the crypts, has been more clearly defined in recent papers. Sakata and Engelhardt (1981) used frozen-section histology to investigate the thickness, compactness and histochemical nature of this layer in rats, mice and guinea pigs (Sakata and Engelhardt, 1979; 1981). The thickness in the proximal colon was found to vary from 182 ± 170 μm in mice to 30 ± 28 μm in guinea pigs. In the distal colon it was much thinner (33 ± 18 μm to 16 ± 7 μm) and more compact. No luminal mucin layer was observed in the caecum. Histochemical analysis showed that luminal mucin was composed of neutral and sialo-mucin types in the proximal colon but also contained sulphated mucin in the distal colon. Differences in stainability by Alcian Blue at pH 1.0 were noted between the luminal and epithelial mucins of the same region. It was suggested that this difference might have arisen due to degradation by the large numbers of bacteria observed in the luminal mucin layer. Much lower numbers of bacteria were observed in the more dense, compact sulpho-mucin layer of the distal colon, suggesting the nature of the mucus may affect the bacterial population.

A further development in technique to observe the luminal mucin layer and its associated bacteria was reported by Rozee et al.
(1982). By using antibodies to crude mucus, the mucus layer in the intestine of mice was stabilised before fixation. The mucus layer and associated bacteria are thus preserved intact. Scanning-electron-microscopy of mouse small intestine preserved by this method shows an unbroken layer of mucin 30-50 μm thick completely occluding the villus surface. Large numbers of coccoid and bacillary bacteria were seen throughout this layer, which was contiguous with the glycocalyx of the microvillus surface. In the young animal, before extensive bacterial colonisation of the gut, the mucus blanket was shown to be very thin (< 10 μm) and fragile. This same observation has been made for the ileum of adult germ-free rats (Davis et al., 1977) suggesting that bacteria may influence mucus secretion.

There have been very few studies on the mucosal flora of man due to the difficulty of obtaining "normal" samples. A study by Nelson and Mata (1970) used tissue sections from surgical patients. Homogenates of gastric and duodenum, from areas that were considered as unaffected by disease, did not contain bacteria at the lowest dilutions tested. Micro-organisms were found in 4 of 7 normal samples of jejunum and in all appendix and colonic specimens. Qualitatively the bacteria found in the tissue samples resembled that in the faeces. Bacteroides and anaerobic Gram positive cocci were among the most numerous found, being cultured in numbers up to 10^8 per gm (wet wt) of tissue.

A more recent study (Edmiston et al., 1982) used samples obtained at colonoscopy to compare the bacterial populations adherent to normal and diseased tissue. A heterogenous community of bacteria
was usually observed with the numbers of bacteria cultured from cancerous tissue, polyp tissue or specimens of inflammatory bowel disease being three orders of magnitude higher than recoveries from adjacent unaffected sites. In contrast to the situation in the rodent, large populations of homogenized bacteria in the mucus layer of adult humans have not been observed.

1.10 Physiological importance of bacteria-mucin interactions

Like the other proposed functions of mucin the possible protective roles for bacterial interactions with mucin have been largely inferred from diverse evidence. One possible function of mucin is to protect the epithelium by providing receptor groups with the same structure as the epithelial cells. Pathogenic viruses, bacteria or toxins may be prevented from reaching the mucosa receptor sites by being bound to, and carried away by, the secreted mucus (Forstner, 1978). Evidence for this is provided by the observations that some pathogenic streptococci can be bound into aggregates by salivary mucin (Williams et al., 1975). Shellfish mucus has been shown to bind pathogenic human viruses via sulphate radicals (Di Girolamo et al., 1977). Pig gastric mucus will bind cholera toxin and prevent its attachment to the cell surface (Strombeck et al., 1974).

A second indirect mode of mucosal protection from pathogens could operate by competitive exclusion. The mucosal surface of the lower gastrointestinal tract, in all normal animals, is heavily colonised by microbial flora (Savage, 1977). Several lines of evidence suggest that this autochthonous flora is important to
the maintenance of health. When germ-free rats or mice are taken from isolators into the normal laboratory they develop severe diarrhoea and usually die. If litter mates are given an enema of faeces or caecal contents from conventional animals, at the time of transfer, these symptoms do not develop (Gustafsson, 1982).

A similar situation exists in the transition after birth of the intestine in germ-free neonate to that of the normal healthy individual with high levels of bacteria. A defined succession of bacteria is seen with initial colonisation of the mucosal coat by autochthonous bacteria that are latter supplanted by other bacteria in the succession (Savage, 1977). However, the role of mucin and mucin fermentation products in influencing the composition of the microbiota has not been defined.

1.11 Evidence that bacteria degrade mucins in vivo

The ability of the enteric flora to degrade and live on gut mucins has been mainly inferred from indirect data. Early evidence was provided by the work of Lindstedt et al. (1965) who found that the faecal material from germ-free rats had six times more hexosamine than that from conventional rats. When the germ-free animals were inoculated with conventional faecal flora, this resulted in a decrease of nitrogen and hexosamine excretion to the level of the conventional animals.

This work was extended and quantitated by Hoskins and Zamcheck (1968). Stools from conventional and germ-free rats fed a residue-free diet were collected and the water soluble fraction dialysed and lyophilized. The mucus from the germ-free animals had high
titres of blood group A and H antigens indicating that the carbohydrate chains were substantially intact. In contrast, these antigens were absent from conventional rat stool mucus. In vitro incubation of the germ-free mucus was carried out using both a complete rat-flora inoculum and a pure culture of *Escherichia coli*. The whole-flora inoculum degraded 75-89% of the hexose and hexosamines after 48 hours anaerobic incubation whereas the pure culture of *E coli* degraded less than half this amount.

Evidence that the faecal flora not only used the blood group glycoproteins, but that it was a selective advantage for them to do so, was inferred from correlating blood group status with faecal blood group-degrading activity (Hoskins and Boulding, 1976). On average there was a 50,000-fold greater population of blood group antigen B-degrading bacteria in blood group B secretors compared to blood group A or H secretors. This implies that the ability to cleave the B antigenic determinant gives bacteria a nutritional advantage in a type B secretor.

Evidence for the in vivo breakdown of mucin in the human colon was obtained by Vercellotti et al. (1977). They extracted the water-soluble contents from the ileum, ascending colon, transverse colon and descending colon of four accident victims. This material was separated into high and low molecular weight components on Sephadex G-100 and each of these peaks was analysed for sugar and protein content. The concentration of the mucin-type sugars fucose, galactose, N-acetylgalactosamine, N-acetylglucosamine and sialic acid was three to ten times lower in the colon than in the ileum. This was interpreted as showing that there was bacterial degradation
of mucin in the colon.

1.12 Studies attempting to quantitate the numbers of mucin-degrading bacteria

Mucin-degrading bacteria in human faecal samples

One of the earliest attempts to enumerate mucin-degrading bacteria was the work of van der Wiel-Korstanje and Winkler (1975) who looked for mucin-degrading bacteria in normal faecal samples and faecal material from ulcerative colitis patients. Pig gastric mucin was incorporated in a brain-heart infusion base and bacterial colonies producing mucinase were detected by flooding the plates with CaCl₂. A mucinase positive result was designated as the production of a clear zone around the colony where the mucin was not precipitated by the CaCl₂. Nearly all the bifidobacteria isolates were found to be mucinase positive. Total enterococci numbers were increased 10 to 100-fold in ulcerative colitis patients but there was also an increase in the numbers and types of enterococci which were strongly mucinase positive. This author believes that the interpretation of their assay is doubtful (see Appendix 2).

More controlled studies have subsequently been done (Miller and Hoskins, 1981; Hoskins and Boulding, 1981; Variyam and Hoskins, 1981). They inoculated commercial pig gastric mucin with serial dilutions of fresh faeces. Mucin degradation was defined as the loss of greater than 25% of the hexose or greater than 30% of the protein. The numbers of mucin-degrading bacteria were then estimated by a "most probable number" technique. Six subjects were tested
over a period of up to nineteen months and the results showed that between $10^7$ and $10^{11}$ bacteria per gram (dry wt) could degrade mucin. Because the numbers of mucin-degrading bacteria were a hundred times and often over a thousand times less than the total number of bacteria, it was concluded that mucin degradation was a function of a specialised sub-population of bacteria.

Further evidence for this hypothesis has come from work with germ-free animals. Gustafsson (1982) serially sectioned normal, frozen rat intestinal wall in the horizontal plane parallel to the mucin layer and picked out sections that contained only organisms in the crypts Lieberkühn. These sections were used to contaminate germ-free animals and from such animals two *Peptostreptococcus sp* were isolated and used to mono-contaminate germ-free animals. One such animal line shows a mucus degradation pattern that is identical to conventional animals. The active species is an oval, Gram-positive, coccus-like organism which occurs singly or in short chains. It is an obligate anaerobe and is maintained only by serial passage in ex-germ-free animals (Gustafsson, 1982).

**Mucin-degrading bacteria in animal studies**

The use of animal models allows a more controlled study as well as proper anaerobic bacterial investigation of material other than faeces. Several studies done on pigs have aimed at enumerating defined bacterial populations by counting the numbers of colonies growing on specific energy sources including pig gastric mucin.

The normal flora of pathogen-free pigs was investigated by Russell (1979) who sampled sites along the large intestine which
were subdivided into luminal contents, luminal surface layer and intestinal wall tissue. Direct microscopic clump counts (DMCC) averaged $13 \times 10^{10}$, $14 \times 10^{10}$ and $5 \times 10^{10}$ per gram (dry wt) respectively for each of these sites. Cultural counts on glucose-cellobiose-rumen fluid agar supplemented with brain-heart infusion gave 56% of the DMCC for the luminal contents and surface layer, but only 20% of the DMCC for the intestinal wall tissue. Over 90% of the bacteria recovered were Gram positive.

Bacterial population differences between the luminal contents and mucosal surface of the pig caecum were also found in a later study by Allison et al. (1979). When pig gastric mucin (2 mg/ml) was added to basal medium the bacterial count was only 13.6% of the total count compared to 54% of the total count for glucose and 0.9% without substrate addition. Bacterial counts using mucin as a substrate were around $3 \times 10^{10}$ per gram (dry wt) in the caecal lumen or around 14% of the total cultural count. The counts from the wall samples, however, gave means of $5 \times 10^{7}$ per cm$^2$ and $0.4 \times 10^{7}$ per cm$^2$ bacteria growing on mucin for the two animals. In the first case this figure was greater than half the maximum recovery of bacteria and in the second it was less than 10% of the maximum recovery of bacteria. The reasons for the variation were not known.

1.13 Mucin degradation by bacteria from culture collections

A major survey of mucin and plant polysaccharide utilisation by intestinal bacteria was carried out by Salyers et al., 1977a,b) using the replicator method of Wilkins and Walker (1975). A
positive result was the ability to ferment the test substrate to a pH less than 6. The substrates included bovine submaxillary mucin, ovomucin and pig gastric mucin, all at 5 mg per ml.

The survey covered 188 strains of Bacteroides and found that none fermented gastric mucin while 22/22 B. thetaiotaomicron strains and 17/24 B. ovatus strains fermented ovomucoid. Only 2/13 B. fragilis type 2393 and 1/19 Bacteroides type "3452A" strains fermented bovine submaxillary mucin. A second survey tested 22 species of the major non-Bacteroides bacteria in the human colon. The only mucin fermented was pig gastric mucin which was utilised by 6/9 strains of Ruminococcus torques and 2/5 strains of Bifidobacterium bifidum.

These results were in contrast to those of Hoogkamp-Korstanje et al. (1979). They measured the optical growth curves of various bacteria growing in a basal medium to which mucin was added. All of the three strains reported, B. thetaiotaomicron NCTC 10582, B. vulgatus NCTC 10583 and B. fragilis NCTC 10584 could utilise gastric mucin.

1.14 Mucin degradation by extracellular enzymes

The production of extracellular glycosidases has been correlated with mucin degradation in a number of papers by Hoskins and co-workers. Enzyme activity was measured using p-nitrophenyl derivatives except for neuraminidase activity which was determined colorimetrically by the method of Warren (1959). They have shown (Hoskins and Boulding, 1981) that mucin degradation by serial dilutions of human faecal bacteria paralleled the production of extra-
cellular glycosidases. Human faecal extracts also significantly degraded pig gastric mucin (Variyam and Hoskins, 1981). Faecal culture supernatants could degrade 55-77% of the gastric mucin hexoses but only 8-20% of the mucin protein as assayed by a modified method of Lowry (Miller and Hoskins, 1981).

Synthetic p-nitrophenyl glycosides were also used by Prizont and Konigsberg (1981) who found high levels of β-N-acetylglucosaminidase, galactosidase and β-N-acetylgalactosamidase in rat caecal content supernatants. These enzymes were capable of degrading the high-molecular-weight components of germ-free rat-caecal contents. They were thought to be of bacterial origin because of the negligible activity found in germ-free animals.

The formation of glycosidases by Bacteroides was investigated by Berg et al. (1978). Of the nine Bacteroides tested, the extracellular glycosidase activities of B. fragilis and B. vulgatus were the highest. B. fragilis NCTC 9343 (ATCC 25285), which is used in this thesis, also produced high levels of α-glucosidase, β-galactosidase and β-N-acetylglucosaminidase. The B. vulgatus B70 was used to investigate the conditions which favoured glycosidase production. Maximum activity was obtained with prerduced proteose-peptone, yeast extract medium containing 1% glucose at pH 7.0. The enzymes were released into the medium in proportion to cell growth but no attempt was made to induce enzyme production using specific substrates.

The study of isolated enzymes capable of degrading mucin carbohydrates has been pursued because their specificity, once known, can be utilised to sequence glycoconjugate chains. It has been found
that some enzymes that can cleave p-nitrophenyl glycosides, or small oligosaccarides, will not act on intact glycoproteins (Brady et al., 1965). This can arise from steric hindrance due to other groups, particularly charged groups, or from a specificity to the glycon portion of the substrate. For example β-galactosidase from Diplococcus pneumoniae will cleave Gal β1 → 4 GlcNAc but not Gal β1 → 3 GlcNAc (Kobata, 1979).

The removal of neuraminic acid appears to be a prerequisite for subsequent mucin degradation (Forstner, 1978) and the production of extracellular neuraminidase is often inducible (Hoffler, 1978). High levels of the enzyme are associated with fresh isolates from pathological material as in the case of Bacteroides sp. (Muller et al., 1970) and substrate specificity is shown towards the various neuraminic acids found on glycoproteins (Kobata, 1979). In human colonic tissues substitution of neuraminic acid by an O-acetyl group at position C4 confers resistance to neuraminidase digestion (Reid et al., 1980). Hindrance to neuraminidase digestion may also arise by the formation of an ester between the carboxylic acid and another hydroxyl on the neuraminic acid or a neighbouring sugar residue (Lev, 1977).

In addition to exoglycosidases, several endoglycosidases have been isolated from bacterial culture-fluid supernatant. Endo-β-acetylglucosaminidase, endo-α-N-acetylgalactosaminidase and endo-β-galactosidase are produced by Diplococcus pneumoniae (Kobata, 1979). These enzymes recognise specific oligosaccaride sequences which are cleaved and will not release monosaccarides. The endo-
α-N-acetylgalactosaminidase is the only purified enzyme known to be capable of hydrolysing the O-glycosidic link between N-acetyl-galactosamine and serine or threonine in mucin-type glycoproteins (Umemoto et al., 1977). Another endo-β-galactosidase has been isolated from *Escherichia freundii* growing on keratan sulphate (Fukuda et al., 1975). This enzyme released GlcNAc-6-sulphate-β1-3 Gal from pig colonic mucin.
CHAPTER TWO

MATERIALS

General laboratory chemicals were of analytical grade and supplied by either Sigma (Sigma Chemical Co., St Louis, Mo.) or BDH (British Drug House, Poole, England) or May and Baker (Dagenham, England). The following chemicals and reagents warrant particular reference.

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Grade or Type</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dyes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acriflavín</td>
<td>neutral</td>
<td>Sigma</td>
</tr>
<tr>
<td>Alcian blue</td>
<td>8 G.S.</td>
<td>Chroma-Gesellscha (Stuttgart - Unterturkheim)</td>
</tr>
<tr>
<td>Basic fuchsin</td>
<td></td>
<td>BDH</td>
</tr>
<tr>
<td>Benzidine HCl</td>
<td>practical grade</td>
<td>Sigma</td>
</tr>
<tr>
<td>Coomassie blue</td>
<td></td>
<td>Sigma</td>
</tr>
<tr>
<td>Enzymes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DNAase I</td>
<td>Type VII</td>
<td>Sigma</td>
</tr>
<tr>
<td>Hyaluronidase</td>
<td>Bovine testes</td>
<td>Sigma</td>
</tr>
<tr>
<td>Neuraminidase</td>
<td>Type V</td>
<td>Sigma</td>
</tr>
<tr>
<td>Trypsin</td>
<td>Type IX</td>
<td>Sigma</td>
</tr>
<tr>
<td>Carbohydrates</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2-deoxyribose</td>
<td>Type IX</td>
<td>Sigma</td>
</tr>
<tr>
<td>Fucose</td>
<td></td>
<td>Sigma</td>
</tr>
<tr>
<td>Galactose</td>
<td>Analar</td>
<td>BDH</td>
</tr>
<tr>
<td>N-acetylgalactosamine</td>
<td>Grade III</td>
<td>Sigma</td>
</tr>
<tr>
<td>N-acetylnuraminic acid</td>
<td>Type VII</td>
<td>Sigma</td>
</tr>
</tbody>
</table>
Chondroitin sulphate: Bovine ligament
Dermatan Sulphate
Mucin: Hog gastric, Type II (Lots 76C-0238, 59C-0197)
Hyaluronic Acid: Type III-S
General
Hexamethyl disilazane
Trimethylchlorosilane
Chromosorb W: AWHP
Agar
Trypticase
Yeast extract

Serva (Feinbiochemica, Heidelberg)
Sigma
Sigma
Sigma
Sigma
Pharmacia Fine Chemicals (Uppsala, Sweden)
Davis Gelatine Co, NZ
BBL (Cockeysville, M.D., USA)
Difco (Detroit, USA)
2.1 Maintenance and storage of bacterial cultures

All bacterial cultures being assessed for mucin-degrading ability were maintained on agar media in which mucin was the major carbohydrate source. This was usually a mucin-based Medium 10 formulation (Table 2.1) containing at least 10 mg/ml of undialysed Sigma gastric mucin. The bacteria were purified and checked periodically by subculture on to agar plates using the anaerobic chamber. For routine work, bacterial cultures were held on agar slopes in butyl rubber-stoppered Kimax tubes at either room temperature or 4°C. Culture viability was between 1 and 3 weeks and subculture was performed as necessary.

Cultures were stored for long periods by freezing the cultures on mucin-containing agar slopes at -80°C, in a Forma ultra deepfreeze (Forma Scientific, Ohio, USA). The original cultures were subcultured no more than yearly, once viability was found to exceed this period.

2.2 Composition of the mucin-containing bacterial growth medium

The basal medium into which mucins were incorporated was based on the formulation of Medium 10 (Caldwell et al., 1966). The usual carbohydrates were replaced with galactose or mucin as required. The volatile fatty acid mixture was originally incorporated in the isolation medium but was later omitted when it was found unnecessary for growth of the isolates selected for study.
<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Yeast extract (Difco)</td>
<td>0.05 g</td>
</tr>
<tr>
<td>Trypticase (BBL)</td>
<td>0.20 g</td>
</tr>
<tr>
<td>hemin solution <strong>b</strong></td>
<td>1.0 ml</td>
</tr>
<tr>
<td>mineral 1 solution <strong>c</strong></td>
<td>3.8 ml</td>
</tr>
<tr>
<td>mineral 2 solution <strong>d</strong></td>
<td>3.8 ml</td>
</tr>
<tr>
<td>volatile fatty acid mixture <strong>e</strong></td>
<td>0.31 ml</td>
</tr>
<tr>
<td>resazurin <strong>f</strong></td>
<td>0.1 ml</td>
</tr>
<tr>
<td>distilled water/carbohydrates solution <strong>g</strong></td>
<td>93 ml</td>
</tr>
<tr>
<td>Na₂CO₃ <strong>h</strong></td>
<td>5 ml</td>
</tr>
<tr>
<td>cysteine <strong>i</strong></td>
<td>2 ml</td>
</tr>
</tbody>
</table>

---

**a.** Based on that of Caldwell and Bryant (1966).

**b.** A stable solution may be prepared by dissolving 100 mg of hemin in 25 ml of ethanol to which 0.28 g KOH has been added. The volume was made up to 100 ml with distilled water. Because of the brown colour, which interfered with turbidometric growth measurements, the quantity of this solution added to the medium can be reduced to 0.1 ml and still supply the requirements necessary for the optimum growth of *B. fragilis.* (Macy *et al.*, 1975)

**c.** Mineral 1: 0.6% K₂HPO₄

**d.** Mineral 2:
- KH₂PO₄ 0.6 g
- (NH₄)₂SO₄ 0.6 g
- NaCl 1.2 g
- MgSO₄·7H₂O 0.25 g
- CaCl₂·2H₂O 0.16 g
- water 100 ml
e. Volatile fatty acid mixture

<table>
<thead>
<tr>
<th>Acid</th>
<th>Volume (ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>acetic acid</td>
<td>17</td>
</tr>
<tr>
<td>propionic acid</td>
<td>6</td>
</tr>
<tr>
<td>N-valeric acid</td>
<td>1</td>
</tr>
<tr>
<td>isovaleric acid</td>
<td>1</td>
</tr>
<tr>
<td>isobutyric acid</td>
<td>1</td>
</tr>
</tbody>
</table>

f. Resazurin was a 0.1% solution in water, shaken before use.

g. For routine maintenance of cultures, undialysed Sigma hog gastric mucin was incorporated at a final concentration of at least 10 mg/ml. When mucin utilisation was being determined in broth, the mucin or other carbohydrates were filter sterilised.

h. Bicarbonate buffer. The solution was prepared in advance by sterilising an 8% Na₂CO₃ solution under CO₂. The flask was opened while hot and bubbled with CO₂ gas until cool. Aliquots of 5 ml were dispensed anaerobically into sterile tubes and stored at room temperature or 37°C until use.

i. Cysteine HCl.2H₂O. A 2.5% (w/v) solution was prepared in advance by autoclaving under N₂ without first neutralising the solution. It was anaerobically and aseptically tubed in 2 ml aliquots and stored at room temperature until use. Formation of crystals in the stored tubes indicated that oxidation to the more insoluble cystine had taken place and such tubes were discarded.
2.3 Preparation and handling of agar media

The formulation of the basal medium is given in Table 2.1. Components were mixed and adjusted to pH 6.8 using 10% NaOH. Mucin was then added in a solid or liquid form and the volume made up to 93 ml with water. After the mucin had dissolved into solution it was transferred to a 100 ml round bottom flask and 1.5 gm of Davis bacteriological agar was added. This mixture was boiled over a bunsen flame and sealed under N₂ when the resazurin (redox indicator) had turned pink. The rubber bung was wired in place and the medium sterilised by autoclaving at 121°C (15 p.s.i.) for 10 min. When the flask had cooled to not less than 50°C it was opened under CO₂ and 5 ml of 8% Na₂CO₃ plus 2 ml of 2.5% cysteine added. Agar slopes were made by anaerobically and aseptically dispensing 3 ml under CO₂ into butyl rubber-stoppered sterile Kimax tubes which were allowed to set on a slope. Tubes of medium that did not completely reduce the resazurin were discarded.

Agar plates were poured anaerobically by entering the bicarbonate-buffered, cysteine-reduced media into the anaerobic chamber at a temperature of not less than 60°C. The vessel stopper was rewired for passage through the vacuum cycle of the entry port. Once inside the flask was opened and poured immediately into plastic petri dishes, which had been depleted of oxygen by equilibration for at least 12 hours in the chamber. Excess water was dried off the lids by 2-3 h exposure to the sterile, dry atmosphere of the chamber and the plates were stored in plastic bags to prevent further moisture loss.
2.4 Mucin sterilisation by filtration

Pig colonic mucin could easily be filtered through a 0.45 μm filter as the preparation had no visible viscosity. In some preparations a fine precipitate was present after redissolving the freeze-dried mucin. This material had to be removed by centrifugation before filtration or flow through the filter was severely impeded.

Sigma gastric mucin however, was very viscous in solution because of its high molecular weight. Other investigators have normally autoclaved this preparation (Salyers et al., 1977a, b; Allison et al., 1979). A dialysed and centrifuged, optically-clear solution could be slowly filtered through a 0.45 μm filter with difficulty. By progressively raising the temperature, it was found that above 40°C, the filtration of up to 50 mls (10 mg/ml) of this gastric mucin was easily achieved. Therefore the medium containing Sigma gastric mucin was filtered at a temperature of between 40° and 45°C.

Initially, sterile mucin solutions, at double strength, were filtered into a sterile vacuum flask and aseptically transferred to the anaerobic chamber. The mucin solution was equilibrated with the oxygen-free atmosphere for at least 2 h in shallow conical flasks. The basal medium was then added at double strength (1:1) and the medium aseptically dispensed into sterile Kimax tubes.

The filtration procedure was later altered by dissolving the mucin into the basal medium without the addition of hemin to the solution. Because it is only soluble in alcohol, hemin solution
was filtered through the sterile filter first, followed by the medium. The bicarbonate solution was then added and the broth slowly bubbled with oxygen-free carbon dioxide for at least 20 min. Cysteine was added to the bulk medium immediately before it was anaerobically and aseptically tubed.

2.5 Maintenance of anaerobic conditions outside the anaerobic chamber

All anaerobic procedures carried out in the laboratory were performed using techniques originally developed by Hungate (Hungate, 1950) to prepare media and cultivate bacteria without exposure to oxygen. The basis of the methods used in this investigation are detailed by Hungate (Hungate, 1969) and the "Anaerobe Laboratory Manual" (1977) 4th Ed. (Virginia Polytechnic Institute and State University, Virginia, USA. [Edited by Holdeman L.V., Cato, E.P. and Moore, W.E.C.]).

Vessels or culture tubes that were opened were kept anaerobic and sterile by a continuous stream of oxygen-free gas. The gas maintained a barrier to oxygen entering the system by providing a constant, oxygen-free outflow from the vessel. It was introduced into the vessel through either a sterile cotton-wool plugged pipette or a sterilised Luerlock syringe with a filter of cotton-wool in the barrel and a shortened, bent No.18 gauge needle on the end. The needle could be flame-sterilised between use and a gas flow rate of about 1 litre per minute was found to be satisfactory for small (< 2 cm) openings.

Gas from commercial grade cylinders was mixed in desired proportions of H₂, N₂, and CO₂ by monitoring the flow through
calibrated gas flow-meters. All traces of oxygen were removed from the mixture by passage through a column of electrically-heated copper filings, on which oxygen is reduced to black copper-oxide. The column was regenerated, as necessary, by hydrogen which reduces the oxide back to bright copper.

2.6 Use of the anaerobic chamber

A Coy anaerobic chamber (Coy Laboratory Products, Ann Arbor Mich., USA) was used in accordance with the manufacturer's instruction manual. The basic methods and operating procedures have been described by Aranki et al., (1969). The chamber was a flexible vinyl box (213 x 81 x 102 cm high) with two pairs of glove inserts. The sides and top were 0.5mm pressed, polished vinyl and the bottom 1.0mm frosty vinyl. A layer of black polythene film covered by a laminated sheet gave further protection to the bottom of the chamber.

Anaerobic conditions (< 5ppm oxygen) were maintained inside the chamber by the use of two catalyst boxes. A fan at the base of each box circulated the chamber atmosphere through two vertically stacked stainless steel trays with wire gauze bottoms. The top tray had a layer of Type D palladium-coated alumina pellets to remove any oxygen by reaction with hydrogen. The lower tray contained a layer of 6-14 mesh activated-coconut charcoal. This was necessary to absorb any sulphide gas generated from cultures which would bind to and inactivate the palladium. The palladium catalyst is sensitive to high humidity. This was counteracted by keeping trays of indicating silica gel inside the chamber. The silica gel was regenerated by heating when necessary.
The palladium was also reactivated to remove sulphide or water, by heating to between 160° and 170°C for 2 h or more.

An atmosphere of 95% $N_2$ and 5% $H_2$ was maintained in the chamber. Hydrogen was consumed by reaction with oxygen which slowly diffused through the vinyl sides. The concentration had to be readjusted periodically, particularly if the chamber was not in active use. The hydrogen concentration in the chamber was determined by taking a sample in a foil-lined gas sampling bag. The percentage hydrogen was then determined by the Department of Paediatrics, University of Auckland Medical School, Auckland, by using a gas-liquid chromatograph set up for breath-hydrogen analysis. Hydrogen was then added to the anaerobic chamber to give a final concentration of 5% (v/v).

All samples were entered into the chamber through a 30 x 46 cm aluminium entry port with clear plexiglass rubber-sealed doors on either end. The entry port was evacuated to 78 cm of Hg and refilled twice with oxygen-free nitrogen. Porous substances such as silica gel were equilibrated with 100% nitrogen for 1-2 h inside the lock in order to reduce the level of oxygen entrapped inside the material. A third evacuation was performed, and the entry port brought to atmospheric pressure with a mixture of 95% $N_2$ and 5% hydrogen. The inside door could then be opened.

Manipulations inside the chamber were carried out through two sets of glove inserts. A pair of cotton or silk liner-gloves was worn over the hands to reduce the difficulty of putting on, or taking off, the neoprene gloves. The latter acted as the barrier to oxygen. A third pair of canvas or soft kid-leather gloves was
worn over the neoprene gloves to protect them from damage. These outer gloves were sterilised when required by conventional steam sterilisation of the canvas gloves or by gamma irradiation of the leather gloves (Appendix 4).

Sterile manipulations could be performed in the hood. The atmosphere was continuously filtered through a bacteriological filter of the type used to maintain germ-free animal isolators. An incandescent flaming device was constructed from a coiled length of nichrome wire and electrically heated when required by activating a foot switch.

Agar plates were stored in the hood in plastic bags, and used as necessary. They were inoculated and removed from the chamber inside conventional polycarbonate Gas-pak jars\(^a\) (BBL Microbiology Systems, Cockeysville, Md.). When required the atmosphere inside the jars was changed by displacement, using the correct gas mixture from the heated copper column. In all cases 5% hydrogen was utilised with fresh palladium catalyst inside the jar to remove any oxygen present. If the jar was closed inside the chamber and removed to the incubator it was

Footnote:

a The jars were later modified by replacing the normal plastic lids with brass ones. Hair-line cracks in the plastic lids of some jars resulted in a slow loss of anaerobic conditions as judged by the resazurin in the media.
possible to incorporate the palladium inside a small glass beaker\textsuperscript{b}. A layer of charcoal was kept separated from the palladium by two layers of tissue paper. Autoclave indicating tape (Propper Manufacturing Co., Long Is., New York) was used inside and outside the jar to detect the presence of hydrogen sulphide which turned the indicating strips on the tape black. By including the tape inside the glass beaker, below the charcoal layer, it was possible to detect hydrogen sulphide poisoning of the palladium catalyst. The charcoal was effective in absorbing the sulphide for periods of greater than one month.

Footnote:

\textsuperscript{b} Closing the jar outside the chamber could potentially result in ignition of the charcoal when hydrogen was introduced to react with the oxygen.
2.7 Gel filtration chromatography

The mucins were characterised by chromatography through Sepharose gels. Types 2B CL, 4B, 4B CL and 6B were used. The gel was poured and packed with a low pressure head (< 20 cm H₂O) and then equilibrated at approximately 30 cm of water, with the pressure being maintained constant by a Mariotte flask. A Pharmacia 60 x 1.9 (I.D.) cm column was used with a bed height of approximately 40 cm of all chromatographic runs. Flow rates of between 3-6 and 5-10 ml h⁻¹ were achieved for the Sepharose 4B and 4B CL columns respectively. Samples of 0.2 to 1.0 ml volume were applied by aspiration through the inlet tube and the column was run in an ascending manner. The running buffer was varied according to need and details are recorded in the results sections. Sodium azide (0.02% w/v) was used to prevent bacterial growth unless the OD₂15nm was to be monitored. All columns were run at 4-8°C, and 2 ml fractions were collected by dropwise mode on an Isco Golden Retriever model fraction collector (Instruments Specialities Co., Nebraska, USA). They were held at 4°C until analysed.

No difference was found between the separating properties of Sepharose 4B and 4B CL. The cross-linked gels, however, gave up to double the flow rates and did not compact under the operating pressures used. Blue dextran dissolved in 1 M NaCl was monitored spectrophotometrically to determine the excluded volume of the gels which was at tubes 10-11. The included volume was located by the increased conductivity of the NaCl and was always in tubes 32-34.
2.8 Extraction of pig gastric and pig colonic mucins from fresh mucosal scrapings

Colonic mucus was collected from pigs of variable age and sex within 20 minutes of death. Approximately 60 cm of colon was excised from the apex of the centripetal and centrifugal coils and faecal material flushed out with cold water. The colon was then slit longitudinally and the mucosal surface scraped with a glass microscope slide to collect the mucus gel (Marshall and Allen, 1978). Gastric mucus was similarly collected after the mucosal surface of the stomach had been gently cleaned with cold, running water. The scrapings were stored in flasks kept immersed in ice-water and processing was commenced immediately after transport back to the laboratory.

Colonic mucus was dispersed in 2 volumes of 0.1 M NaCl containing 10 mM sodium phosphate, pH 7.0, by making two passes with the pestle in a motor-driven Thomas teflon-pestle tissue grinder. The mucus was centrifuged at 10,000 x g for 10 min, and the insoluble material resuspended in buffered NaCl of equivalent volume to the discarded supernatant. A second homogenisation and centrifugation step was carried out to remove any remaining soluble proteins and enzymes.

The gastric mucosal scrapings and the insoluble colonic mucus were then processed in an identical manner. Colonic mucus was dissolved in 4 volumes of 0.1 M NaCl, 0.1 M sodium phosphate, 0.02% NaN₃ and 0.4 M β-mercaptoethanol pH 7.5. Gastric mucus was homogenised in 1 volume of double strength buffer. Both mucins were held at 0°C under N₂ for 12 h.
Collections of mucosal scrapings that were heavily contaminated by bacterial and mucosal cells had to be heated to inactivate extracellular enzymes and then treated with DNAase. The high content of DNA appeared to form a gel that remained after mercaptoethanol treatment and co-precipitated mucin on centrifugation. To avoid this the mucins were heated at 80°C for 30 min and then incubated at 37°C for 6 hours with 25 μg of DNAase I per gram (wet weight) of original mucus.

Mucins solubilised by the mercaptoethanol treatment were recovered by centrifugation at 10,000 x g for 10 min and the supernatant exhaustively dialysed against distilled water. The solution was then further digested with DNAase I (25 μg/g original wet weight of mucus) at 37°C for 6 h in 0.1 M sodium phosphate buffer 0.01 M MgCl₂, 0.01% NaN₃, pH 7.0. Trypsin (25 μg per g wet weight) was then added and the digestion continued for 12 h. An equivalent amount of trypsin was again added and digestion continued for another 12 h. The solution was then exhaustively dialysed against distilled water, at 4°C. Insoluble material was removed by centrifugation at 10,000 x g for 10 min and the supernatant freeze-dried for storage.
2.9 Colorimetric determination of hexose and fucose by the anthrone reaction

The concentration of hexose and methylpentose (fucose) was measured using the anthrone reagent (Bailey, 1958). Bailey (1958) showed that hexoses and fucose have a greater extinction coefficient at 0.05% (w/v) anthrone concentration than pentoses, while the opposite is the case when the anthrone concentration is 0.01% (w/v). Only crude mucosal scrapings produced significant colour that could not be attributed to furfural derivatives of hexose or fucose on the basis of their absorption spectra. This is probably due to a combination of nucleic acids and tryptophan-containing proteins (Spiro, 1966). Determinations were therefore carried out using the 0.05% (w/v) anthrone concentration.

Anthrone reagent. Anthrone reagent was prepared by mixing 70 ml of conc. sulphuric acid (d = 1.84) into 30 ml of water. Anthrone (0.05 gm) was added while the acid was still hot and the reagent was kept on ice for at least 2 h but no more than 24 h.

Procedure. Aliquots of 5.0 ml of reagent were pipetted into boiling tubes standing in ice. Samples of 0.5 ml were layered on top of the reagent and the reaction started by mixing on a rotamixer. The tubes were immediately placed in a boiling water bath for exactly 7 min, cooled in an ice-water mixture, and allowed to stand for 30 min. The absorption was measured at 625 nm against a reagent blank containing only water or buffer.
Standards of 0.01% and 0.02% galactose, and 0.01% and 0.02% fucose were included with every run. Fucose gave approximately twice the molar extinction coefficient of galactose.

2.10 Determination of free neuraminic acid

The procedure was adapted from the method of Warren (1959).

Reagents. Sodium metaperiodate (0.2 M) was dissolved in 9 M phosphoric acid. Sodium arsenite (10% w/v) was dissolved in 0.1 N $\text{H}_2\text{SO}_4$ with 0.5 M sodium sulphate. Thiobarbituric acid (0.6% w/v) was made up in 0.5 M sodium sulphate. All solutions required warming to dissolve them. The first two are stable for several months in stoppered bottles at room temperature. Thiobarbituric acid solution was kept no longer than one week.

Procedure. A sample volume of 0.1 ml was mixed with 0.1 ml of the periodate solution and reacted for 20 min at room temperature. Excess periodate was then destroyed by the addition of 0.5 ml of sodium arsenite solution, which initially forms a yellow-brown precipitate that dissolves on further mixing. Failure to form this colour indicated a lack of excess periodate and the sample was repeated with fresh periodate solution or a lower sample concentration. When the sample had decolorised, 1.5 ml of thiobarbiturate solution was added, rotamixed, and the tube heated in a boiling waterbath for exactly 15 min. The tubes were cooled in tap water for 5 min and the pink chromophore extracted into 2 ml of cyclohexanone by vigorous mixing on a rotamixer. The top organic phase was clarified by brief centrifugation on a bench centrifuge and the optical density read at 532 nm and 549 nm.
The procedure produced a linear response curve and was quantified by the inclusion of neuraminic acid standards (Sigma Type VII from human urine) with every run. With crude or semi-purified mucus samples the presence of 2-deoxyribose necessitated the use of a 2-deoxyribose standard as well. This sugar forms a chromophore with a maximum absorption at 532 nm as opposed to the free neuraminic acid which forms a chromophore with a maximum absorption at 549 nm. By reading both wavelengths and using the molar extinction coefficients determined with every run, the relative quantities of 2-deoxyribose and neuraminic acid were calculated by simultaneous equation.

If samples contained mercaptoethanol or other reducing reagents it was necessary to dialyse them before oxidation with periodic acid. Quantitative acid hydrolysis of bound neuraminate was not possible in samples with strong buffering capacity. The high sensitivity of the assay, however, usually meant that dilution was necessary and thus the acid concentration could readily be obtained.

**Release of bound neuraminic acid**

In order to measure total neuraminic acid, the bound neuramininate was released by either enzymic or acid hydrolysis. *Clostridium perfringens* neuraminidase (Sigma Type V) at 0.5 mg per ml was incubated with the sample for 3h at 37°C in 0.1 M citrate-phosphate buffer, pH 5.0. Acid hydrolysis was carried out in 0.05 M H₂SO₄ at 80° for 1 h. In a comparative trial of the two methods, using pig colonic mucin as the substrate, the acid hydrolysis was found to release 20-25% more neuraminic acid,
as determined by the thiobarbiturate method.

The optimum hydrolysis time for neuraminate was determined and found to be 1 h ± 10 min. Acid hydrolysis destroyed the neuraminate at a rate of 7% per h but also released 2-deoxyribose from any DNA present. The occurrence of a chromophore with an absorbance maximum at 532 nm was indicative of the presence of DNA but was not quantitative under the hydrolysis conditions used. Conditions for the quantitative analysis of DNA by this procedure have been determined by other workers (Gold and Shochat, 1980).

2.11 Carbohydrate analysis by GLC (Bhatti, Chambers and Clamp, 1970)

Hydrolysis of mucins. Total carbohydrates were measured after the oligosaccharides had been hydrolysed by methanolysis. This gave quantitative recoveries of the sugars as the 1-O-methyl-monosaccharide.

Preparation of dry methanol. Commercial grade methanol was dried and distilled before use by refluxing 500 ml of methanol with 2.5 gm of magnesium turnings and 0.1 gm of iodine for 1 h. The solution was then distilled to collect the methanol (b.p. 65°C) in a clean, dry container.

Preparation of methanolic HCl. HCl gas was produced by dropping $H_2SO_4$ ($d = 1.84$) onto $NH_4Cl$. Eight grams of $NH_4Cl$ were used per 300 ml of dry methanol. The reaction required heating to maintain a constant rate of gas production. The HCl was dried by bubbling through an $H_2SO_4$ ($d = 1.84$) trap and finally dissolved into the
methyl alcohol by passage through a sintered-glass sparger. The concentration of HCl was followed by titration against a standard Na₂CO₃ solution using a few drops of a 0.1% solution of methyl orange as the indicator. The methanolic HCl was made to a concentration of 1.5 N and was aliquoted in 250 ml flasks that were sealed with teflon-lined caps. Reagent that was not required for immediate use was stored at 4°C because of the slow formation of methyl chloride, which resulted in the loss of half the HCl every 3 to 4 months at room temperature. The methanolic HCl was discarded when the acid strength had decreased below 0.75 M HCl.

Preparation of silver carbonate. All steps were performed in low light. AgCO₃ was precipitated by slowly adding Na₂CO₃ (2.65 g in 30 ml of H₂O) to AgNO₃ (8.6 g in 100 ml of H₂O) over a period of 10 min. The precipitate was filtered off, washed with acetone then air-dried and stored in a dark brown bottle.

Methanolysis. The samples were prepared by pipetting up to 0.1 ml of culture media or standard solution of mucin, containing no more than 1 mg of mucin, into a 2 ml hydrolysis vial. The internal standard of 0.1 μmol of meso-insitol was then added and the vials briefly centrifuged to force the liquid to the bottom of the vial. They were then freeze-dried and held over phosphorous pentoxide for at least 12 h. The hydrolysis was carried out by adding 0.5 ml of methanolic HCl to the vial which was bubbled with N₂ gas for 30 sec and immediately sealed. The vials were placed in an oven at 85°C for 24 hours after which they were cooled and broken open. The residual acid was neutralised by adding a small
amount of solid AgCO₃. This was titurated and then pH tested by spotting on pH paper (range 4-6). When enough AgCO₃ had been added to neutralise the solution, the amino-sugars were re-N-acetylated by adding 0.1 ml of acetic anhydride. The solution was mixed and the vial covered with parafilm for at least 6 h. Vials were centrifuged briefly on a bench centrifuge to clear the supernatant which was transferred into a clean vial. The precipitate was washed twice by additions of 0.5 ml of dry methanol followed by titration and centrifugation. Pooled supernatants were cooled on dry ice, evaporated to dryness under vacuum and stored over phosphorous pentoxide.

To test for free sugars the solutions were not hydrolysed but the monosaccarides were extracted from the freeze-dried material, containing the meso-inositol internal standard, by washing three times with dry methanol. Pooled supernatants were evaporated to dryness under vacuum.

The methanolysis procedure was adhered to, even for non-acetylated sugars, as acetic anhydride is required for quantitative recovery of the methylglycosides from the AgCO₃ precipitate (Clamp, 1977).

Derivatisation. The trimethylsilytating reagent was made fresh daily by mixing 0.2 ml of trimethylchlorosilane, 0.2 ml of hexamethyldisilazane and 1 ml of dry pyridine that had been stored over KOH. The reagent was kept in a teflon-stoppered vial sealed with parafilm around the outside. To make the derivative, 0.2 ml of the reagent was pipetted into the sample vial, which was then sealed with several thickness of parafilm.
The vial was mixed thoroughly to dissolve the carbohydrates and left for 30 min at room temperature before injecting 1-3 μl onto a 78 cm glass column (0.13 cm I.D.) packed with OV-101 on Chromosorb W. The temperature was held at 140°C for 5 min and then programmed to increase to 220°C at 0.5° per min. A Varian 1240 gas chromatograph was used in single column mode with a flame ionisation detector.

Quantitation. The chromatograms were analysed by comparing the peak areas of each sugar to the peak area of the internal standard. Molar response curves for both the free and the methylglycosidic sugars were determined and this factor was used to calculate the μmoles of sugar present in the original sample.

2.12 Periodic acid/Schiff (PAS) colorimetric assay of glycoproteins

The Sepharose column runs were conveniently monitored by the method of Mantle and Allen (1978).

Reagents. Schiff reagent was prepared by dissolving 1 gm of basic fuchsin in 100 ml of boiling water. The solution was cooled to 50°C and 20 ml of 1 M HCl added. Impurities were removed by treating the solution twice with activated charcoal followed by centrifugation. The reagent was stored in the refrigerator. Immediately before use 0.1 gm of sodium metabisulphite was added to every 6 ml of Schiff reagent and the solution was incubated until it was clear or light yellow in colour (1-2 h). The Schiff reagent was retreated with charcoal if a background colour was left after bisulphite reduction.
Periodic acid solution was made fresh each time by dissolving periodic acid crystals in an equivalent (w/v) volume of water. The acid was diluted to 0.2% (w/v) by mixing 20 μl with 0.7 ml of acetic acid and 9.3 ml of water.

Procedure. The assay detected between 5 and 200 μg of glycoprotein in a total volume of 2 ml. Carbohydrates were oxidised at 37°C with 0.2 ml of the 0.2% periodic acid solution. Colour was developed by adding 0.2 ml of the Schiff reagent and leaving it for at least 30 min. The optical density was read at 555 nm and the assay semi-quantified by constructing a calibration curve for every set of data using a known amount of the same glycoprotein preparation as that being assayed.

The assay was found to be incompatible with reducing agents such as mercaptoethanol as they interfere with the periodic acid oxidation. Sodium chloride concentration up to 1 M showed no interference but the use of Tris buffer resulted in a high background.

An investigation into the specificity and the nature of the groups detected by this method is reported in Chapter three.

2.13 Colorimetric assay of glycoproteins using Alcian blue

The Alcian blue assay was adapted from that of Hall et al., (1980). Alcian blue is a basic dye that can cross-link polyanionic molecules forming insoluble complexes. These complexes are washed free of excess stain and the bound dye resuspended and measured photometrically.
Reagents. Alcian blue 8 G.S. was made up as a 0.1% (w/v) solution in either 0.1 M sodium acetate/acetic acid buffer each containing 0.025 M NaCl, pH 5.8, or in 0.1 M HCl, and stored for no longer than one week. The dye was suspended and insoluble material carefully removed by centrifugation at 3500 r.p.m. on a bench centrifuge immediately before use. The washing solution was 40% (v/v) ethanol in the same sodium acetate buffer, pH 5.8. The resuspending solution was 10% (w/v) SDS in water.

Procedure. A 1.0 ml sample containing less than 200 µg of mucin was mixed with 0.5 ml of Alcian blue solution in a 2 ml microcentrifuge tube. The tubes were incubated at room temperature overnight and insoluble complexes precipitated by centrifugation at 10,000 g for 4 min. The excess dye was carefully removed with a Pasteur pipette and the insoluble complex washed with the ethanol-buffer solution. After a second centrifugation and washing step the Alcian blue was redissolved in 1 ml of 10% SDS. The optical density was read at 620 nm and the assay semi-quantitated by constructing a standard curve for every set of data, using a known quantity of the same mucin preparation as that being assayed.

The selectivity and nature of the acidic groups detected by the Alcian blue assay was investigated and the findings are reported in Chapter 3. These studies resulted in the subsequent use of 0.1 M HCl as assay buffer, because results are less likely to be affected by contaminating polyanions at the low pH.
2.14 Determination of sulphate content

Introduction

Sulphate determinations were originally done by the gelatin/barium chloride method of Clark et al., (1971). Values of 1-2% of the mucin freeze-dried weight were obtained but the daily fluctuations and non-linearity of the standard curve were observed. The benzidine method of Antonopoulos (1962) was found to be very reproducible and gave repeatable linear standard curves in the region of 5-100 μg/ml sulphate.

Reagents Benzidine (0.5% w/v) was dissolved in 95% ethanol and centrifuged before use. Benzidine is a known carcinogen and was handled appropriately. Thymol (0.5% w/v) was dissolved in 2 M NaOH.

Procedure All glassware was washed in 0.1 M HCl and rinsed with distilled water before use. Mucins and standard sulphate solutions were hydrolysed for 24 h in 1.0 ml of 25% formic acid at 100°C using 2 ml sealed glass ampoules. A 0.3 ml aliquot of hydrolysate was mixed in a micro-centrifuge tube with 0.5 ml of 95% ethanol, 0.2 ml of benzidine reagent and 0.5 ml of amyl alcohol. The tubes were left to precipitate on ice for at least 60 min and then centrifuged at 10,000 x g for 5 min. Excess benzidine was removed by washing the precipitate twice with 1 ml of 50% acetone, 25% amyl alcohol and 25% ethanol. The precipitates were recentrifuged at 10,000 x g for 5 min each time.
Benzidine sulphate was redissolved by carefully washing the outside of the tube in distilled water and dropping it into a boiling tube containing 5 ml of 0.6 N HCl. After vigorous rotamixing the benzidine was diazotised at room temperature using 1 ml of 0.1 N NaNO₃ followed by 5 ml of thymol reagent. The optical densities of the tubes were read at 505 nm and unknowns calculated from a six-point standard curve of up to 100 μg per ml sulphate. The standard curves closely matched those of Antonopoulos (1962) indicating that quantitative precipitation of sulphate was achieved.
CHAPTER THREE

MUCIN CHARACTERISATION

Introduction

Physical and chemical analysis of the mucins was initially carried out to confirm that the mucin preparations were similar to those of other workers. Having established this fact, the same methods were then used to examine the bacterial degradation products. These studies revealed marked differences between the bacterial degradability of colonic and gastric mucins and further investigations were then undertaken in an attempt to find a structural basis for the differences. The results of the physical and chemical studies of the mucin preparations are summarised in this chapter.

3.1 Ultracentrifugation of the mucins after PAS staining

Introduction

Most mucins show a strong dependence of sedimentation coefficient upon concentration because the expanded nature of the glycoprotein molecule leads to molecular interactions at relatively low (> 1 mg per ml) concentrations (Gibbons, 1966). This is corrected for by determining sedimentation constants for several concentrations of mucins and extrapolating to determine the value at infinite dilution. Jabbal, Forstner et al., (1975) used PAS-stained mucins for band ultracentrifugation. Except for the fuschin attached to sialic and fucose residues this modification did not alter the immunological, chemical or physical
parameters of rat goblet cell mucin. The strong colour of the stain allowed detection of the glycoproteins at less than 0.2 mg per ml which is essentially at infinite dilution where no molecular interactions are taking place. Sedimentation – velocity analysis can therefore be done using multiple determinations made during a single run. A modification of this method was used to examine the homogeneity of the mucin preparations used in this thesis.

Method

The glycoproteins were stained by adapting the procedure used in the colorimetric assay of Mantle and Allen (1978). The final periodate concentration was increased ten times to 0.1% and the glycoprotein concentration was at 2 mg per ml. This is the same periodate concentration as that used by Jabbar et al., (1975). A sample of 0.5 ml of the stained mucin solution was carefully layered onto 34.6 ml of sucrose in a linear gradient from approximately 17 to 45% (w/w) sucrose. A batch of gradients was prepared in 50 ml polyallomer centrifuge tubes using the method of Baxter-Gabbard (1972) by slowly freezing and thawing a 30% (w/w) sucrose solution. They were stored frozen until required and then thawed overnight in a refrigerator. Centrifugation was carried out at 25,000 r.p.m. for 23 h at 20°C in an SW27 Sorval rotor. Fractions of 2.0 ml were collected from the bottom of the tube by displacement with mineral oil. The optical density of each fraction was read at 555 nm and the refractive index determined using an Abbe refractometer, Type 1 (Atago Co. Ltd., Japan).
Results and discussion

The results obtained are graphed in Fig. 3.1. Colonic mucin gave a single disperse peak. Approximately two-thirds of the gastric mucins, however, precipitated out of solution during the run. Therefore the results shown for these mucins are only of the fraction which remained in solution and may not be a true representation of the total preparation. The gastric mucin that was prepared from mucosal scrapings, and that remained soluble, sedimented as a unimodal peak more slowly than the colonic mucin. Sigma mucin that remained in solution showed a continuous increasing concentration down the tube indicating that the preparation was very heterogenous with respect to molecular weight.

The reason for the precipitation of the gastric mucins was not investigated but some preparations of intestinal mucin have also been observed to precipitate on PAS treatment (A. Wesley, Personal communication).

Using the data obtained sedimentation coefficients were calculated by the method of Martin and Ames (1961). This method and the results obtained are discussed in Appendix five.

In conclusion the colonic mucin sedimented as a single, unimodal, though disperse peak after being stained by the PAS procedure. Gastric mucin that stayed in solution also showed a single peak, but the Sigma gastric mucin was poly-disperse and of higher molecular weight than the other preparations.
Figure 3.1  Density gradient centrifugation of PAS labelled gastric and colonic mucins

Colonic (□—□), gastric (△—△) and Sigma gastric (●—●) mucins were labelled by PAS staining and layered onto sucrose density gradient tubes as detailed in section 3.1. After 23 h centrifugation at 25,000 rpm in a SW27 Sorval rotor, the concentration of mucin was measured by the optical density at 555 nm in 2 ml fractions along the tube.
3.2 Cellulose acetate electrophoresis

Introduction

Cellulose acetate electrophoresis of pig gastric mucin has been reported by Snary and Allen (1971), dog gastric mucin by Pamer et al., (1968), and tracheal mucin by Gallagher et al., (1975). It is a useful method for separating high-molecular-weight polymers on the basis of charge. When used in conjunction with the Alcian blue stain it can also be used to detect as little as 20 µg per ml of glycosaminoglycans (Breen et al., 1970). Therefore it was used to examine the charge homogeneity of the mucins, as well as to check for contamination by other negatively charged polymers.

Method

Electrophoresis was done according to the method of Breen et al., (1970) on a Gelman Microzone system (Gelman Instruments Co., Mich., USA). Samples of 0.5 µl were loaded along with standards of chondroitin sulphate, hyaluronic acid and dermatan sulphate at 0.4 mg per ml. Good visualisation of mucin samples was found to require a concentration of at least 5 mg per ml. A sample of water-soluble gastric mucin was prepared from freeze-dried, dialysed mucosal scrapings which were suspended at 50 mg per ml in water and 2 ml centrifuged at 10,000 x g for 10 min. The supernatant was evaporated to a gel (approx 20–40 mg per ml) under vaccuum before application onto the strips.
All samples were run in unbuffered 0.2 M zinc sulphate. The strips were stained with 1% Alcian blue in 0.1 M sodium acetate buffer, pH 5.8. Duplicate strips were also stained by adapting the PAS procedure of Mantle and Allen (1978). The strips were oxidised with 80% ethanol in water containing 0.01% (w/v) periodic acid at 37°C for 2 h. This solution was then replaced with fresh 80% ethanol and 10 ml of Schiff reagent added for every 100 ml of the ethanol solution. The Schiff reagent was prepared by the method of Mantle and Allen (1978). After 30 min the strips were washed in 95% ethanol and the results recorded.

Results obtained using Alcian blue staining

Colonic and gastric mucins showed single, diffuse bands running at 0.7 - 1.0 and 0.5 - 0.8 respectively, relative to the origin and the hyaluronic acid standard. Chondroitin sulphate ran at a position of 1.4 and dermatan sulphate at a position of 1.2 relative to the hyaluronic acid standard. The Sigma gastric mucin showed a light very diffuse track in the region of the other mucins but there was also a major band in the same position as the hyaluronic acid standard. Undigested, water-soluble pig gastric mucin ran as a single band at a position of 0.5 - 0.8 relative to hyaluronic acid.

Results obtained with PAS staining

All colonic mucin bands stained with both the PAS and Alcian blue stains. In contrast all the gastric mucin preparations also showed a PAS positive, Alcian blue negative band at a position of 0.2 - 0.3 relative to the hyaluronic acid standard. A control
sample of Sigma oyster glycogen gave a single PAS positive, Alcian blue negative band also at 0.2-0.3 relative to the hyaluronic acid standard. The Sigma mucin and gastric mucin preparation left sharp bands at the origin which showed no diffusion and are probably from insoluble material in the samples.

**Effect of cetyl pyridinium chloride precipitation**

The gastric mucin preparation that had been digested with trypsin was fractionated with cetyl pyridinium chloride (CPC) as detailed in Section 3.10. The precipitated fraction showed only one band. This band stained with Alcian blue and PAS in the same position as the unfractionated sample. Mucin that was not precipitated by CPC gave an Alcian blue and PAS positive band at a position of 0.5-0.6 relative to the hyaluronic acid as well as a PAS positive but Alcian blue negative band at 0.2-0.3 relative to hyaluronic acid.

**Discussion and conclusions**

No glycosaminoglycans were detected in samples prepared from mucosal scrapings. Sigma mucin, however, contains hyaluronic acid.

Colonic mucin migrated as one disperse band which was stained by PAS and Alcian blue. Sigma gastric mucin, gastric mucin prepared by digestion with trypsin and gastric mucin prepared without digestion all showed a PAS positive, Alcian blue negative band in addition to Alcian blue staining material. This band was not precipitated by CPC and migrates in the same position as glycogen.
It is therefore a neutral or near neutral species of glycoprotein and appeared to be the predominate band in Sigma gastric mucin. Mucin obtained from fresh mucosal scrapings was different from the commercial preparation because of the greater quantity of Alcian blue staining acidic mucins that were present.
3.3 Digestion of the mucin preparations with hyaluronidase

Introduction

The results of the cellulose acetate electrophoresis indicated that the Sigma gastric mucin preparation was contaminated by hyaluronic acid. This contamination could account for the decreased response of the Sigma mucin in the Alcian blue assay at pH 1.0 compared to pH 5.8. Confirmation of the presence of hyaluronic acid was sought by specific enzymic digestion.

Method

The mucins (10 mg/ml) were digested with hyaluronidase in 0.1 M sodium acetate buffer, 0.15 M KCl, pH 5.5 at 37°C for 4 h using a 1:5 (w/w) mucin to enzyme ratio. The results were quantified by determining the response of samples in the Alcian blue assay at pH 5.8 over 5 mucin concentrations up to a maximum of 100 µg/ml mucin. The slopes of the response curves before and after enzymic digestion were compared.

A sample of the Sigma gastric mucin preparation was also chromatographed on a Sepharose 4B column after hyaluronidase digestion and assayed by the Alcian blue reaction.

Results and Discussion

No decrease in Alcian blue binding was observed after hyaluronidase digestion of the colonic and gastric mucin preparations. The Sigma gastric mucin, however, lost 43% of its Alcian blue response after digestion confirming the presence of hyaluronic acid. When the hyaluronidase treated
Sigma gastric mucin was chromatographed on Sepharose 4B (Fig.3.2) there was no loss of a complete Alcian blue reactive peak. The Alcian blue reactive mucin and hyaluronic acid therefore eluted in overlapping positions. The remaining Alcian blue reactivity may not all be due to acidic mucins, as it was not determined whether the hyaluronidase had completely eliminated the reactivity due to hyaluronic acid.

3.4 Determination of the protein content by the Lowry and Coomassie blue protein assays

Introduction

The Lowry protein assay (Lowry et al., 1951) and modifications of it can quantify as little as 5 µg protein. It is often used to determine the protein content of gastrointestinal mucins. Pamer et al., (1968) noted that the Lowry assay gave low protein values when used with BSA as a standard. A purified sample of dog gastric mucin showed ten times less protein when assayed by the Lowry method compared to a summation of its amino acid content.

Most colour in the Lowry assay is derived from chromogenic amino acids, and the rest is due to specific sequences of peptide bonds that are not yet well characterised. Tyrosine and tryptophan are the main colour-forming amino acids but cystine, cysteine and histidine also contribute (Peterson, 1979).

Mucins contain only trace amounts of aromatic amino acids and more than 50% of these are eliminated by proteolytic digestion along with the cysteine residues (Pearson et al., 1981; Mantle, Mantle et al., 1981). Around 65% of the protein is protected
Figure 3.2  Loss of Alcian blue reactivity from Sigma gastric mucin after digestion by hyaluronidase.

Molecular size distribution on Sepharose 4B of the Alcian blue reactivity of Sigma gastric mucin before (△--△) and after (○--○) digestion with hyaluronidase.

The apparent mucin concentration has been calculated from a standard curve of the Alcian blue reactivity of dialysed, undigested Sigma gastric mucin. Digestion was carried out using bovine testicular hyaluronidase at 2 mg per ml and dialysed Sigma gastric mucin at 10 mg per ml in 0.1 M sodium acetate, 0.15 M KCl, pH 5.5 for 4 h at 37°C. A 0.5 ml sample was applied to the column and Alcian blue reactivity determined at pH 5.8.
from proteolytic digestion by glycosylated serine and threonine residues which, together with proline, comprise over 50% of the amino acid content (Allen, 1981). Thus the Lowry assay, like the optical density at 280 nm, is probably very sensitive to contamination by non-mucin proteins or to loss of the non-glycosylated region of the peptide core. Reducing agents such as mercaptoethanol and cysteine also give a chromophore in the Lowry assay with a spectrum identical to that of protein. This can, however, be corrected for by blocking the sulphydryl groups (Hughes et al., 1981).

A more sensitive assay for proteins relies on the difference spectrum produced when Coomassie blue binds to peptide bonds. This assay can quantitate down to 1 µg of protein (Bradford, 1976).

The Lowry and Coomassie blue assays were tested at different times to ascertain their suitability as mucin protein assays.

**Lowry protein assay**

The modified Lowry protein assay of Markwell et al., (1978) was used to quantify protein in mucin preparations. This procedure incorporates 1% SDS in the alkaline reagent A. Two different preparations of dialysed Sigma gastric mucin gave apparent protein readings of 16% and 17% (w/w) when compared to a BSA standard. This mucin was incorporated into basal medium at 10 mg per ml and a 2.5 mg sample was run on a Sepharose 4B column. The effluent was monitored by the modified Lowry assay and the results are graphed in Fig. 3.4
Figure 3.4  Sigma gastric mucin in basal medium on Sepharose 4B

A sample of 2.5 mls of Sigma gastric mucin (10 mg/ml) in basal medium was applied to a Sepharose 4B column and eluted with 0.1 M NaCl, 0.02 M sodium phosphate pH 7.0. Fractions of 2 ml were collected and monitored by the PAS reaction (□—□), the Alcian blue assay at pH 5.8 (△—△), and the modified Lowry assay of Markwell et al. (1978) (○—○).
All the colour detected was in the total column volume and not in the high-molecular-weight mucin fractions. Therefore the bacterial degradation of the mucin proteins could not be determined by assaying the Sepharose columns using the Lowry method.

Miller and Hoskins (1981) published a modified Lowry method to give improved colour development in mucins. This assay was tested by adapting the SDS method of Markwell et al., (1978). The sample was added to reagent A and heated at 70°C for 45 min. After cooling reagent B was then added and the conventional procedure continued. In a comparative trial, mucins were treated in duplicate by both the usual procedure of Markwell et al., (1978) and the modified procedure of Miller and Hoskins (1981). Protein concentrations were determined from standards treated in a like manner.

By heating the sample in reagent A the apparent protein content of dialysed Sigma gastric mucin increased from 17% (w/w) to 36% (w/w), that of the colonic mucin preparation increased from 10% (w/w) to 26% (w/w), and that of the gastric mucin extracted in the laboratory increased from 22% (w/w) to 35% (w/w) protein. Duplicates agreed to within 8% but, while the optical densities of the mucins were increased approximately 20% by heating the samples in reagent A, the response of the BSA dropped with this treatment. This caused the high percentage increases in apparent protein content.
The modified method of Miller and Hoskins (1981) was applied to analyse dialysed Sigma gastric mucin that had been run on a Sepharose 4B column. Figure 3.5 shows that approximately a third of the detected colour could be attributed to low molecular weight non-dialysable peptides. The mucin protein content was therefore overestimated because of this contamination but higher values were obtained than with the normal Lowry assay.

**Coomassie blue protein binding assay**

The method of Bradford (1970) was tried on two mucin preparations. Sigma gastric mucin and pig colonic mucin were tested at 1 mg/ml in duplicate against a BSA standard of 0-100 μg per millitre. The results showed an apparent protein content of 8% (w/w) in the gastric mucin and 5% (w/w) in the colonic mucin. These values are at least 50% below the expected values assuming that no non-mucin protein was present. It was therefore thought that the carbohydrate groups prevented the dye from binding to the peptide core of the mucin.

An attempt was made to improve the binding by mild alkaline hydrolysis of the O-glycosidic linkages to the peptide core. Solutions of pig colonic mucin and Sigma gastric mucin at 1 mg per ml were incubated in 0.1 M NaOH at 37°C. A BSA standard was similarly treated at 100 μg per ml. Samples of 0.1 ml were removed at 0, 12, 17 and 42 h and the response determined in the micro-Coomassie blue assay of Bradford (1976).
A sample of 10 mg of dialysed Sigma gastric mucin was eluted from a Sepharose 4B CL column with 0.1 M NaCl, 20 mM sodium phosphate buffer, pH 7.0. Fractions of 2 ml were collected and 0.5 ml used in the PAS assay (△—△). Protein was determined by the modified Lowry assay of Miller and Hoskins (1981). A 1 ml sample was heated with 3 ml of reagent A at 70° C for 45 min. After cooling, 0.03 ml of reagent B was added and the sample left at room temperature for 10 min. It was then mixed with 0.3 ml of phenol reagent and the optical density determined at 660 nm after 45 min (○—○).
The sodium hydroxide caused an immediate 20% increase in the response of the BSA standard. No significant change then occurred in the next 42 h. Sigma gastric mucin and pig colonic mucin slowly lost 6% and 20% respectively of their reactivity over the 42 h period.

The alkali treatment was probably effective in cleaving carbohydrate chains off the peptide core but destruction of serine and threonine may have also occurred. (Neuberger, Gottchalk et al., 1972). This would eliminate the binding sites of Coomassie blue on the peptide backbone. The assay is therefore not suitable for use in determining the protein content of mucins as it is probable that only non-glycosylated mucin peptides and protein contamination are detected.

3.5 Effect of periodic acid concentration on the PAS reaction of mucins

Introduction

Pearse (1978) lists conflicting results that have been obtained with the PAS procedure by different investigators. The stainability of mucosubstances varies with the use of different periodic acid concentrations and reaction times. In order to interpret results obtained by the procedure of Mantle and Allen (1978) the assay was carried out on various sugars using two different periodate concentrations.

Method

The procedure of Mantle and Allen (1978) was followed with periodic acid concentrations of 0.01% and 0.2% being used in the oxidising step.
Results and discussion

A summary of the optical densities produced by each acid concentration is given in Table 3.2 The PAS colorimetric assay is used to quantitate less than 0.2 mg per ml of glycoprotein. Individual components of the mucins used in bacterial experiments therefore are at concentrations of less than 0.02 mg per ml which are ten times lower than the concentrations used in this test. Only 2-deoxyribose showed significant colour development in this concentration range with 0.01% periodic acid.

At the higher 0.2% periodic acid concentration all monosaccharides and polysaccharides formed intense colour. The use of this acid concentration might locate mucins in the Sepharose column fractions which do not stain in the assay of Mantle and Allen (1978). This modification, however, would be subject to greater interference from contaminating sugars and may not be linear in response.

In conclusion the PAS procedure of Mantle and Allen does not detect mucin monosaccharides, maltose or glycosaminoglycans at 0.01% (w/v) periodic acid.

3.6 Effect of pH on Alcian blue binding to mucins

Introduction

Lev and Spicer (1965) showed that Alcian blue dye will bind to sulphate and carboxyl groups at pH 2.5. However, at pH 1.0 only sulphate groups are ionised and can bind the dye. Therefore, using this criterion, an attempt was made to determine the
<table>
<thead>
<tr>
<th>Sugar</th>
<th>Concentration</th>
<th>Optical density</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mM</td>
<td>mg/ml</td>
<td>0.01% I_4^-</td>
<td>0.2% I_4^-</td>
</tr>
<tr>
<td>2-deoxyribose</td>
<td>0.63</td>
<td>0.84</td>
<td>&gt; 3</td>
<td>0.105</td>
</tr>
<tr>
<td>fucose</td>
<td>5.0</td>
<td>0.82</td>
<td>0.062</td>
<td>0.911</td>
</tr>
<tr>
<td>galactose</td>
<td>5.0</td>
<td>0.90</td>
<td>0.071</td>
<td>0.957</td>
</tr>
<tr>
<td>inositol</td>
<td>5.0</td>
<td>0.90</td>
<td>0.045</td>
<td>0.731</td>
</tr>
<tr>
<td>N-acetylglucosamine</td>
<td>0.5</td>
<td>0.110</td>
<td>0.062</td>
<td>1.297</td>
</tr>
<tr>
<td>NeuNac</td>
<td>5.0</td>
<td>0.14</td>
<td>0.273</td>
<td>1.389</td>
</tr>
<tr>
<td>maltose</td>
<td>0.27</td>
<td>0.1</td>
<td>0.071</td>
<td>1.269</td>
</tr>
<tr>
<td>hyaluronic acid</td>
<td>-</td>
<td>0.5</td>
<td>0.150</td>
<td>3.54</td>
</tr>
<tr>
<td>chondroitin sulphate</td>
<td>-</td>
<td>1.0</td>
<td>0.293</td>
<td>3.05</td>
</tr>
</tbody>
</table>

---

a Using the procedure of Mantle and Allen (1978)

b Where necessary these were determined after dilution into 0.2% (w/v) sodium metabisulphite.
relative contribution of the mucin sulphate and neuraminate groups to Alcian blue binding.

Method

Alcian blue staining of the gastric and colonic mucins was determined over a range of pH values. A 0.1 M sodium acetate/acetic acid buffer was used for pH 5.0, 4.5 and 4.0 and a 0.1 M glycine/HCl buffer for pH 3.5, 3.0 and 2.0.

In a second experiment the staining intensity at pH 5.8 was compared to that at pH 1.0 (0.1 M HCl) for several negatively charged polymers. The Alcian blue binding capacity was determined at each pH by doing a linear response curve over six concentrations up to 100 μg/ml. DNA, hyaluronic acid and chondroitin sulphate were assayed at concentrations up to 10 μg/ml.

Results and discussion

In the first experiment the Alcian blue binding capacities of the gastric and colonic mucin preparations did not alter from pH 5.0 down to pH 2.0. In the second experiment chondroitin sulphate showed a decrease in Alcian blue binding capacity at pH 1.0 compared to pH 5.8 (Table 3.3). The other polymers showed losses in staining intensity of 20% for the colonic and gastric mucin preparations, 31% for the Sigma gastric mucin, 57% for the hyaluronic acid and 66% for the DNA solution. At pH 1.0 the Sigma gastric mucin, the hyaluronic acid and the DNA formed fine, powder-like sediments which were distinctly different from the heavy gel-like precipitates of the other mucins and chondroitin sulphate.
<table>
<thead>
<tr>
<th></th>
<th>Conc.</th>
<th>pH 1.0</th>
<th>pH 5.8</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sigma gastric mucin</td>
<td>100 µg/ml</td>
<td>1.20</td>
<td>1.74</td>
</tr>
<tr>
<td>Gastric mucin</td>
<td>&quot;</td>
<td>1.63</td>
<td>2.03</td>
</tr>
<tr>
<td>Colonic mucin</td>
<td>&quot;</td>
<td>3.19</td>
<td>3.91</td>
</tr>
<tr>
<td>Hyaluronic acid</td>
<td>10 µg/ml</td>
<td>0.76</td>
<td>1.77</td>
</tr>
<tr>
<td>Chondroitin Sulphate</td>
<td>10 µg/ml</td>
<td>1.94</td>
<td>2.22</td>
</tr>
</tbody>
</table>

\(^a\) The optical densities were determined as detailed in Section 3.6
Alcian blue binding to negative charges other than sulphate was reduced but not eliminated, by doing the assay at pH 1.0. The staining of the gastric and colonic mucins decreased only 20% and is therefore probably mainly due to sulphate residues. Sigma gastric mucin was known to contain hyaluronic acid (Section 3.2) and therefore the lower staining intensity at pH 1.0 was expected.

The results were further confirmed by treating the Sigma gastric and colonic mucins with *Clostridium perfringens* neuraminidase. More than 80% of the neuraminic acid (by method of Warren, 1959) was removed from the colonic mucin but the Alcian blue binding capacity at pH 5.8 of the Sigma gastric and the colonic mucin was not affected.

3.7 **Effect of NaCl on Alcian blue binding to glycoprotein**

**Introduction**

The concept of critical electrolyte concentration (C.E.C.) was originated by Scott *et al.*, (1965). Electrolytes such as NaCl or MgCl₂ can compete with cationic dyes like Alcian blue for the anionic sites on the mucins. The critical electrolyte concentration is the highest concentration at which dye-substrate staining is still observed. Sulphate containing mucosubstances will generally stain at higher (> 0.2 M) molarities than those with carboxyl groups. The C.E.C. of the mucins was examined to get information on their Alcian blue binding properties.
Method

The effect of increasing NaCl concentration on the Alcian blue assay was determined by incorporating NaCl into the normal acetate buffer, pH 5.8 containing 25 mM MgCl₂. The NaCl concentration in the assay tube (including the Alcian blue) ranged from 0 to 1.0 M NaCl in steps of 0.1 M. After overnight incubation the precipitate was centrifuged down and washed using the normal assay procedure and sodium acetate buffer. A sample of 0.1 mg of mucin in 1 ml was used for each assay and controls of hyaluronic acid and chondroitin sulphate were also included at 0.01 mg per ml.

Results and discussion

All the polymers showed a maximum staining intensity at 0.1 M NaCl (Table 3.4). Chondroitin sulphate did not lose any Alcian blue binding capacity up to 1.0 M NaCl. Hyaluronic acid showed a rapid decrease to 0.5 M NaCl but then retained a high background of 39% of the maximum staining intensity up to 1.0 M NaCl. The Alcian blue binding capacity of the mucins decreased sigmoidally from 0.1 M NaCl to approach a minimum value of 10% of their binding capacity at 1.0 M NaCl.

The initial increase in the binding of Alcian blue is thought to be due to a reduction in dye-dye charge repulsion in the presence of electrolytes (Pearse, 1968). Interpretation of the critical electrolyte concentrations of the mucins was not possible because the mucins, which have sulphate esters, showed only slight binding of Alcian blue at 1.0 M NaCl, while the binding of Alcian blue to the chondroitin sulphate was not affected.
Table 3.4  Effect of NaCl on Alcian blue binding

<table>
<thead>
<tr>
<th></th>
<th>Conc.</th>
<th>0.1 M NaCl</th>
<th>1.0 M NaCl</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sigma gastric mucin</td>
<td>200 µg/ml</td>
<td>1.10</td>
<td>0.192</td>
</tr>
<tr>
<td>Gastric mucin</td>
<td>&quot;</td>
<td>1.30</td>
<td>0.153</td>
</tr>
<tr>
<td>Colonic mucin</td>
<td>&quot;</td>
<td>2.03</td>
<td>0.224</td>
</tr>
<tr>
<td>Hyaluronic acid</td>
<td>20 µg/ml</td>
<td>1.14</td>
<td>0.44</td>
</tr>
<tr>
<td>Chondroitin sulphate</td>
<td>&quot;</td>
<td>1.24</td>
<td>1.22</td>
</tr>
</tbody>
</table>

a Values given are the maximum and minimum observed at pH 5.8 for concentrations of NaCl between 0 and 1.0 M.

b Experimental details are given in Section 3.7
In contrast hyaluronic acid, which does not have sulphate groups, retained 39% of its binding capacity at 1 M NaCl. Therefore factors such as the number and orientation of the charges must also be important to the binding of Alcian blue.

3.8 Sepharose chromatography of the mucins

Introduction

Extensive use was made of the Sepharose gels to examine the molecular size and composition of the mucins. The fractionation ranges for polysaccarides in the gels are:

- Sepharose 2B \(10^5\) - \(20 \times 10^6\)
- 4B \(3 \times 10^4\) - \(5 \times 10^6\)
- 6B \(10^4\) - \(1 \times 10^6\)

The cross-linked (CL) forms have substantially the same porosity. ("Gel filtration, theory and practice" Handbook by Pharmacia Fine Chemical Co., Uppsala, Sweden).

Mucin-type glycoproteins, however, are highly expanded and linear in nature (Allen, 1981). Therefore mucins are excluded from Sepharose gels at much lower molecular weights than polysaccarides or proteins.

Method

The methods used in the Sepharose chromatography are detailed in Chapter two and variations are noted in the legends to the figures.
Results

The colonic mucin always gave a unimodal peak on Sepharose 4B (Fig. 3.6). It eluted near the excluded volume of the gel with slight tailing of the material in the included volume. PAS and Alcian blue assays of colonic mucin had identical elution profiles. By collecting the high-molecular-weight fractions (tubes 20-25) and the low molecular weight fractions (tubes 26-35) from 10 mgs of material, a dry weight analysis was done. Seventy-one percent of the non-dialysable freeze-dried weight was found to elute in the high molecular weight region. Thirty-four percent of the starting material was found in the low-molecular-weight region and is presumed to be non-dialysable peptides and nucleic acid fragments. The extra material recovered is probably bound salts and water.

Colonic mucin was also run on Sepharose 2B CL and produced a symmetrical peak around tube 22 which is half-way into the included volume of the gel (data not shown). Colonic mucin is therefore a subunit of the high-molecular-weight, undegraded molecule found by Marshall and Allen (1978).

The dialysed Sigma gastric mucin preparation was run on Sepharose 4B (Figure 3.7) and Sepharose 2B CL (Figure 3.8). An excluded, PAS reactive and Alcian blue negative peak separated out on both gels but there was also a pronounced included PAS peak on the Sepharose 2B CL. Alcian blue reactivity occurred as a shoulder in the region of the PAS included peak but the main Alcian blue binding peak was in the lower-molecular-
Figure 3.6  Sepharose 4B of colonic mucin

A sample of 2.5 mg of the colonic mucin preparation was eluted from a Sepharose 4B column with 0.1 M NaCl, 50 mM phosphate buffer pH 7.0. Fractions of 2.0 ml were collected and 0.5 ml assayed for PAS (□□□□) reactivity and 0.25 ml assayed for Alcian blue binding (▲▲).
Figure 3.7  Sigma gastric mucin on Sepharose 4B CL

A sample of 10 mg of dialysed Sigma gastric mucin was eluted from a Sepharose 4 B CL with 0.1 M NaCl, 50 mM phosphate buffer, pH 7.0. Fractions of 2.0 ml were collected and 0.25 ml assayed for PAS (Δ—Δ) and Alcian blue (●—●) reactivity.
Figure 3.8  Sigma gastric mucin on Sepharose 2B CL

A sample of 10 mg of dialysed Sigma gastric mucin was applied onto a Sepharose 2B column and eluted with 0.1 M NaCl, 50 mM phosphate buffer pH 7.0. Fractions of 2.0 ml were collected and 0.25 ml of each was analysed for PAS (△△) and Alcian blue (●●) reactivity. The PAS reactive mucin in the excluded volume did not contain any Alcian blue binding material.
weight region. Some of this is due to hyaluronic acid (Sections 3.2 and 3.3). When fractions 10 to 25 of the Sepharose 4B column were pooled and dialysed they contained 84% of the freeze-dried weight and fractions 26-35 contained 21% of the freeze-dried weight of the starting material.

Sigma gastric mucin therefore consists of an undegraded, or aggregated, very high-molecular-weight fraction and lower-molecular-weight material. It is likely that the lower-molecular-weight mucins result from protease digestion of higher-molecular-weight material as described by Starkey et al., (1974). However, when Sigma gastric mucin was trypsin digested, using the same protocol as for the extraction of the colonic mucin, only half the excluded fraction on Sepharose 4B was shifted into the included volume (data not shown). This is in contrast to the results obtained with fresh mucosal scrapings which are readily degraded using mercaptoethanol and trypsin. The molecule could be inherently resistant to cleavage by trypsin or prior mercaptoethanol treatment is required to allow access of the enzyme. This latter possibility was not tested but treatment with 0.5 M mercaptoethanol for 2 h at pH 8.6 followed by iodoacetamide was less successful than trypsin treatment in degrading the excluded peak on Sepharose 4B (data not shown).

Gastric mucin prepared by mercaptoethanol treatment and trypsin digestion of fresh mucosal scrapings had both PAS and Alcian blue reactivity. When run on Sepharose 4B (Fig. 3.9) or 2B CL (Fig. 3.10) the peaks of Alcian blue and PAS reactivity were always separated by 1 to 4 tubes. This
Figure 3.9  Gastric mucin preparation on Sepharose 4B CL

A sample of 10 mg of the gastric mucin preparation was run on the Sepharose 4B CL column and eluted with 0.1 M NaCl, 20 mM phosphate buffer, pH 7.0. Fractions of 2 ml were collected and 0.25 ml used in the PAS (▼▼) and Alcian blue assay (●●). Alcian blue reactivity was determined at pH 1.0.
Figure 3.10  Gastric mucin preparation on Sepharose 2B CL

A sample of 10 mg of gastric mucin isolated from fresh mucosal scraping by mercaptoethanol treatment and tryptic digestion was applied onto a Sepharose 2B CL column. The sample was eluted with 0.1 M NaCl, 20 mM phosphate buffer pH 7.0 and fraction of 2.0 ml were collected. A 0.25 ml sample of each was analysed for PAS (○—○) and Alcian blue (■—■) reactivity. The small peaks in the eluted volume were from fractions that had a slight cloudy haze and may be from aggregated, insoluble mucin.
difference was repeatable in more than six determinations and was not abolished when 1 M NaCl was used as the eluting buffer. Therefore this preparation was further investigated and has been fractionated into two mucins using cetyl pyridinium chloride precipitation (section 3.10).

When the soluble fraction of freeze-dried, pig gastric mucosal scrapings was run on Sepharose 2B CL (Fig. 3.11) the Alcian blue and PAS reactivities peaked in the excluded volume. Cellulose acetate electrophoresis confirmed that no glycosaminoglycans were present in this preparation and it had been treated with DNAase I eliminating high-molecular-weight DNA. Therefore the Alcian blue reactivity of gastric mucin is derived from a very high-molecular-weight mucin.

3.9 Effect of mild acid hydrolysis of the mucins on the PAS and Alcian blue reactions

Introduction

Pig colonic mucin gave twice the response (w/w) of gastric mucin in the Alcian blue assay, but the inverse was true for the PAS assay (data not shown). Other investigators have noted an inverse relationship between the PAS reaction and sulphate content (Pamer et al., 1968, Spee-Brand et al., 1980). Published glycoprotein sequences (Slomiany et al., 1980, Slomiany et al., 1972) indicate that the non-reducing terminal sugars of gastric and colonic mucin carbohydrate chains are the main or only periodate oxidisable residues. One explanation for the inverse
Figure 3.11  Undegraded gastric mucin on Sepharose 2B CL

A sample of 10 mg of freeze-dried gastric mucosal scrapings was suspended in 1 ml of 0.1 M NaCl, 10 mM phosphate buffer pH 7.0. The insoluble material was centrifuged off by 10,000 × g for 5 min and the supernatant applied to a Sepharose 2B CL column. The column was eluted with the same buffer as the sample and fractions of 2 ml were collected. Samples of 0.5 ml were analysed for PAS reactivity (▼▼) and Alcian blue binding capacity (●●) at pH 1.0.
PAS and Alcian blue staining could be that sulphate esters on the C3 or C4 of the non-reducing terminal carbohydrate residue block periodate oxidation. Studies by Slomiany et al., (1972) and Liau et al., (1982) on gastric mucin, however, found that most sulphate occurred as hexosamine-6-sulphate. Only minor amounts of hexosamine-disulphate were found.

An acid stability study of the reactive groups in the PAS and Alcian blue assays was carried out to characterise the type of group being detected.

Method

The mucins (2 mg/ml) were hydrolysed in 0.25 M HCl at 100°C using a boiling water bath. Samples of 0.1 ml were removed every 10 min and immediately diluted to 1.0 ml with 0.1 M HCl. Their abilities to react in both the PAS and Alcian blue assays were determined. Control samples were heated for the same length of time in 0.05 M phosphate buffer pH 7.0 and diluted into 0.1 M HCl for colorimetric determination.

Results

The mucins that were heated at pH 7.0 did not lose their reactivity in either the PAS or Alcian blue assays. Hydrolysis of the Alcian blue reactive groups is graphed in Figure 3.12 as the log of optical density versus time. The decrease in reactivity shows approximately first order kinetics from 10 to 60 min. Half-lives of the Alcian blue reactivity calculated from the slopes gave values of 23, 27 and 30 min for the Sigma gastric mucin, the gastric mucin preparation and the colonic mucin respectively.
Hydrolysis of the PAS reactive groups is similarly graphed in Figure 3.13. The colonic and gastric mucins show a large, very rapid initial decrease in PAS reactivity. This initial phase occurs to a lesser extent in the Sigma gastric mucin.

Discussion

Alcian blue staining

The rate of acid hydrolysis of Alcian blue reactivity is similar in all three mucins suggesting that the same reactive group is probably present. Rees (1963) showed that sulphate esters of carbohydrates could be characterized by their stability to acid hydrolysis. Three distinct categories of acid stability are found. Sulphate esters can be on equatorial hydroxyls, axial hydroxyls or primary hydroxyl groups with half-lives of 0.1 - 0.4 h, 0.4 - 1.45 h and 1.5 - 2.4 h respectively for hydrolysis in 0.25 M HCl at 100°C. The half-lives obtained by the Alcian blue assay would indicate that equatorial hydroxyl sulphate groups are necessary for the binding of the dye.

N-acetylglucosamine-6-sulphate was the major species found in pig gastric mucin (Slomiany et al., 1972) but the acid conditions they used for isolation of sulphated oligosaccharides would have hydrolysed most of any equatorial sulphate esters present. All hydroxyl groups of glucosamine are in an equatorial position and thus disulphated glucosamine should be rapidly converted to glucosamine 6-sulphate by acid hydrolysis.

A second possibility that could account for the data is that the Alcian blue assay was decreased due to release of the sugar-
sulphate as a monosaccharide. Liao and Horowitz (1982) hydrolysed rat gastric mucin labelled with radioactive sulphate at 100°C for 1 h in 0.25 M H₂SO₄. They found that 36% of the sulphate was recovered as the inorganic ion and only 26% was released as low molecular weight sugar sulphate. Together these comprise only 62% of the mucin sulphate present in the unhydrolysed sample. Pamer et al., (1968) hydrolysed sulphated glycoprotein from dog gastric mucosa for 2 h at 100°C in 0.1 N HCl. They found that 92% of the sialic acid, 89% of the fucose, 54% of the hexose, 31% of the hexosamine and 28% of the sulphate was released in this period and could pass through a dialysis membrane. In contrast the results shown here indicate that only 11-16% of the Alcian blue reactivity was left when the pig colonic and gastric mucins were hydrolysed in 0.25 M HCl at 100°C for 1 h. This rate of loss is much faster than that found by Pamer et al., (1968) or Liao et al., (1982) for the release of sulphate from gastric mucin glycoprotein.

The sulphate hydrolysis results of other workers could be equated with Alcian blue hydrolysis if the Alcian blue data shows the loss of only labile sulphate groups in secondary sulphate positions. Therefore the remaining sulphate could still be bound as the more stable 6-sulphate ester. An alternative to this hypothesis would be that glucosamine-6-sulphate residues are located in two postions. Sulphated groups near the non-reducing end of the carbohydrate chains may react better with Alcian blue than sulphated groups near the reducing terminus in the interior of the molecule. Outer chain sulphated groups would also be cleaved off as monosaccarides faster than internally located residues.
Figure 3.12 Effect of mild acid hydrolysis on the PAS and Alcian blue reactions of the mucins

Legends are on the opposite page.
If sugars were cleaved sequentially from the non-reducing terminal this might account for the comparatively stable Alcian blue reactivity in the first 10 min of hydrolysis.

A corollary to these hypotheses would be that an Alcian blue negative reaction in gastric mucin does not necessarily indicate that sulphate esters are not present. Direct assay of mucin sulphate and sugars during acid hydrolysis would be required to verify the hypothesis.

PAS staining

The slopes in Figure 3.13 show an initially rapid rate of hydrolysis indicating the loss of very labile groups. This initial loss of reactivity is greatest in the gastric and colonic mucin preparations which have higher neuraminate contents than the Sigma mucin (Table 3.5). Jabbal et al., (1975) demonstrated that 40% of the PAS staining intensity was lost when sialic acid was dialysed away after neuraminidase hydrolysis of small intestinal mucin. The rest of the staining reaction was thought to be due to fucose. However when the mucins used in this thesis were stained before and after neuraminidase hydrolysis, without dialysis, the PAS reactivity increased slightly (data not shown). The difference may be because the periodate concentration used (0.01%) was ten times lower than that used by Jabbal et al., (1975). At this concentration free neuraminic acid does not form significant colour in the reaction (Section 3.5), and the bound neuraminic acids constitute less than 4% (w/w) of the total composition (Table 3.5). Small intestinal mucin, as used by
Jabbal et al., (1975) has up to 18% (w/w) sialic acid.

Pamer et al., found that 89% of the fucose was hydrolysed from dog gastric mucin at 100°C for 2 h in 0.1 N HCl. Free fucose is not detected by the PAS assay but bound fucose was shown to be labelled by Jabbal et al., (1975). Therefore the initial loss of PAS reactivity is probably due to a combination of neuraminate and fucose hydrolysis.

The results of Pamer et al., (1968) also show that galactose is slowly released by mild acid hydrolysis. The ability of bound galactose to react in the assay has not been determined but it could account for the slower, second phase of the PAS hydrolysis curve. Hexosamines, however, cannot react in the assay, either as the free or bound sugar, because of the low periodate concentration used. The hydrolysis of the outer, more labile, residues should have created terminal hexosamine groups, but no increase in PAS reactivity was observed. If the hexosamine residues were periodate blocked by terminal, Alcian blue-reactive, secondary sulphate esters then the data (Fig. 3.12) shows that greater than 80% of such groups would have been hydrolysed after one hour. Section 3.5 shows that hyaluronic acid is not PAS reactive at the periodate concentration used providing further evidence that hexosamines are not oxidised in the assay.

In conclusion periodate-Schiff reactivity arises from acid-labile terminal groups such as neuraminic acid, fucose and possibly galactose. The decrease in PAS reactivity during
hydrolysis is because free monosaccarides do not react in the assay.

The Alcian blue reactivity was more labile than would be expected for hexosamine 6-sulphate groups. Comparisons with the data of other workers indicates that at least 40% of the sulphate groups should have still been bound to the glycoproteins yet only 11 to 16% of the Alcian blue reactivity remained. These results suggest that some of the sulphate esters do not react with the Alcian blue dye.
3.10 Fractionation of gastric mucin with cetyl pyridinium chloride (CPC)

Introduction

Attempts were made to separate the Alcian blue and PAS reactivities of the gastric preparation by anion exchange chromatography on DEAE cellulose. This was only partially successful. A predominately PAS positive fraction did not bind to the column and a predominately Alcian blue positive fraction was eluted at 0.3–0.4 M NaCl. Greater than 80% of the mucin, however, appeared to co-elute at 0.1 to 0.2 M NaCl without showing significant separation of the Alcian blue and PAS reactivities.

Slomiany and Meyer (1972) had isolated sulphated glycoproteins from the pig gastric mucosa using CPC precipitation. As CPC binds to glycoproteins in an analogous way to that of Alcian blue this method was applied to the gastric mucin preparation to try and separate the PAS and Alcian blue fractions that were observed on the Sepharose columns.

Method

Gastric mucin was dissolved in 0.1 M NaCl at 10 mg per ml. The exact amount of CPC required to precipitate acidic mucins was determined in a trial by sequentially adding 0.50 μl aliquots of CPC to a 1 ml sample of the mucin. After every 2 or 3 additions the solution was centrifuged at 10,000 x g for 3 min in order to determine if further precipitation occurred when CPC was added. The bulk solution was then treated by adding
0.35 ml of 1% CPC per 10 mg of mucin and incubating the mixture at room temperature for 30 min. Acidic mucins were separated out by centrifugation at 2,000 x g for 5 min and the precipitates redissolved in the original volume of 1 M NaCl. Ice cold 100% ethanol was then added to both the supernatant and precipitate fractions to a final concentration of 80% (v/v) ethanol. Precipitated mucins were recovered by centrifugation at 2,000 x g for 10 min on a bench centrifuge and then washed in a further volume of ethanol. The CPC-free fractions were then dissolved in a minimum volume of distilled water and freeze-dried for storage. Characterisation of the fractions was done using the methods previously described.

Results and discussion

The analysis of each fraction is given in Table 3.5. Around 75% of the Alcian blue binding capacity and 43% of the dry weight was precipitated by the CPC but 65% of the PAS reactivity and 57% of the dry weight was left in solution. When the fractions were chromatographed on Sepharose 4B (Fig.3.14) the mucin that had been precipitated by CPC eluted slightly ahead of the fraction that was not precipitated by CPC. Therefore, CPC separated the gastric mucin into two major fractions which are different in molecular size as well as in their PAS and Alcian blue reactivities. However the sulphate content (w/w) of each fraction was almost identical (Table 3.5). Duplicate analyses of sulphate content agreed to within 6%. These results imply that the Alcian blue binding capacity does not reflect the sulphate content of these mucin fractions.
Figure 3.13 A & B  Gastric mucin fractionated by CPC precipitation

Legends are on the opposite page.
The same method was also applied to fractionate the colonic mucin preparation. Approximately 0.7 ml of 1% CPC was required to precipitate 10 mg of colonic mucin. Only 5% of the PAS reactivity was left in the supernatant demonstrating that colonic mucin cannot be separated into two major fractions using CPC.

Variyam and Hoskins (1981) used CPC to purify commercial pig gastric mucin (Nutritional Biochemical Division of International Chemical and Nuclear Corporation, Cleveland, Ohio) before use in bacterial degradation studies. They added 1.8 mg of CPC per mg of mucin hexose. Only 5% of the hexose could then be centrifuged out in the precipitate suggesting that their commercial mucin was similar to that purchased from Sigma for this investigation. It has been shown previously (Fig. 3.2) that Sigma gastric mucin has only a minor Alcian blue fraction that cannot be attributed to contamination by hyaluronic acid. Most of the Alcian blue reactivity of the gastric mucin preparation used for this investigation was precipitated by CPC.
3.11 Precipitation of mucins by acriflavin

Inoue et al. (1965, 1966) used the turbidity caused by the precipitation of mucins with acriflavin to monitor the elution profile of sulphate esters in chromatographic fractions. The turbidity formed repeatable, linear curves with standard mucins. This method was tested to determine the relative response of the mucin preparations used in this thesis.

Method

The mucins were dissolved at 0.5 mg per ml. A 0.5 ml sample of each was added to 0.1 ml of 0.5% aqueous acriflavin. After 15 min the solution was diluted with 2.5 ml of water and the turbidity measured at 660 nm.

Results and discussion

The colonic mucin, Sigma gastric and the gastric mucin preparation gave optical densities of 0.407, 0.010 and 0.113 respectively. These results do not reflect the sulphate content of these mucins (Table 3.5). However the Alcian blue reactivity of the colonic, Sigma gastric and gastric mucin preparations (pH 5.8, 200 mg/ml mucin) showed optical densities of 3.9, 1.7 and 2.0 (Table 3.4) respectively. This is the same order as the acriflavin readings except that the Sigma gastric mucin, which contains hyaluronic acid, gave proportionately higher Alcian blue readings.

In conclusion the acriflavin method of Inoue et al (1965) is approximately ten times less sensitive than Alcian blue in
measuring sulphate esters. In addition, as with the Alcian blue assay, there is a discrepancy between the sulphate content of the mucins and the relative response of the assay.
3.12 Chemical composition of the mucins

The composition of the mucins fractions is summarised in Table 3.5. Variations of up to 20% in individual sugar components were noted between different batches of extracted mucins. The results given are from the mucin batches on which the reported bacterial degradation studies were done. All analyses were done in duplicate.

Neuraminic acid contents determined by G.L.C. were up to three times higher than those determined by the thiobarbiturate method of Warren (1959). This was also noted by Marshall and Allen (1978). An investigation into the differences showed that it was not due to O-acetylated sialic acids as reported by Culling et al. (1974) as prior alkaline hydrolysis resulted in less than a 5% increase in the optical density produced by the thiobarbiturate reaction. The difference is most likely to be error in determination of the neuraminic acid by gas-liquid chromatography. The long retention time on the column results in a very low (< 0.25) molar response value compared to meso-inositol.
<table>
<thead>
<tr>
<th>Mucin</th>
<th>Fuc&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Gal&lt;sup&gt;a&lt;/sup&gt;</th>
<th>GalNAc&lt;sup&gt;a&lt;/sup&gt;</th>
<th>GlcNAc&lt;sup&gt;a&lt;/sup&gt;</th>
<th>NeuNAc&lt;sup&gt;b&lt;/sup&gt;</th>
<th>SO₄&lt;sup&gt;c&lt;/sup&gt;</th>
<th>Protein&lt;sup&gt;d&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sigma gastric mucin</td>
<td>5</td>
<td>19</td>
<td>24</td>
<td>29</td>
<td>1</td>
<td>1.3</td>
<td>17</td>
</tr>
<tr>
<td>Colonic mucin</td>
<td>8</td>
<td>20</td>
<td>10</td>
<td>23</td>
<td>4</td>
<td>1.3</td>
<td>10</td>
</tr>
<tr>
<td>preparation</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gastric mucin</td>
<td>7</td>
<td>17</td>
<td>8</td>
<td>20</td>
<td>2</td>
<td>1.2</td>
<td>22</td>
</tr>
<tr>
<td>preparation</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CPC precipitated</td>
<td>6</td>
<td>18</td>
<td>6</td>
<td>18</td>
<td>2</td>
<td>1.1</td>
<td>-</td>
</tr>
<tr>
<td>gastric mucin</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CPC soluble gastric</td>
<td>6</td>
<td>14</td>
<td>8</td>
<td>21</td>
<td>0</td>
<td>1.0</td>
<td>-</td>
</tr>
<tr>
<td>mucin</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> Method of Shin<sub>bi</sub> et al. (1970)

<sup>b</sup> Method of Warren (1959)

<sup>c</sup> Method of Antonopoulos (1962)

<sup>d</sup> Method of Markwell et al. (1976)
3.13 Summary and conclusions

The gastric and colonic mucins used in the thesis have been shown to be similar in composition to those of other workers listed in Table (1.1). As the mucins were used in bacterial degradation studies it was important that the carbohydrate was as undegraded as possible. This was reflected in the high neuraminate and fucose content. The presence of non-mucin protein, however, was not critical as Trypticase was added to the bacteriological media.

Treatment of mucins by mercaptoethanol, trypsin and DNAase does not result in the loss of mucin carbohydrate but the products were shown to be subunits of the native high molecular weight mucins by both ultracentrifugation and Sepharose chromatography. Cellulose acetate electrophoresis, hyaluronidase digestion and comparative Alcian blue analysis showed that only Sigma gastric mucin was contaminated with Alcian blue reactivity due to glycosaminoglycans.

Investigation of PAS staining by the method of Mantle and Allen (1978) demonstrated that bound acid-labile sugars such as sialic acid and fucose account for most of the colour reactivity. Alcian blue binding, though, is mainly due to sulphate groups and not neuraminic acid. However, this Alcian blue reactivity of the mucins did not quantitatively reflect their sulphate content.

A combination of CPC fractionation, cellulose acetate electrophoresis and Sepharose chromatography was used to
demonstrate that gastric mucin, but not colonic, can be separated into at least two fractions.

Slightly under half the dry weight of the gastric mucin preparation was precipitated by CPC and did not contain a separate neutral mucin band on electrophoresis. Neutral mucin remaining in the supernatant was not isolated free from Alcian blue reactivity and gave two bands on electrophoresis. Therefore the relative content of the neutral and acidic species could not be accurately quantified in the gastric mucins.
CHAPTER FOUR

UTILISATION OF MUCINS BY BACTERIA

4.1 Growth of Bacteroides fragilis on Sigma gastric and colonic mucin

Introduction

The study by Salyers and Vercellotti et al. (1977) did not detect any B. fragilis type I or type II strains (Johnson, 1978) that were capable of degrading Sigma gastric mucin. Bacteroides fragilis strains ATCC 25285 and ATCC 23745 were therefore included in the original experimental format to act as negative controls and as a check on cultural procedures. However, it was quickly observed that they grew well on basal medium plus Sigma gastric mucin but very poorly on basal medium alone. Further studies were done to determine the extent of mucin degradation by these type strains of bacteria.

Method

Both strains of Bacteroides were maintained between experiments on basal medium plus Sigma gastric mucin. They were subcultured more than 4 times on this mucin media before use. An inoculum was taken from a freshly grown agar slant and suspended in 2 ml of basal medium containing 10 mg per ml gastric or colonic mucin. The tubes were incubated for 24 h at 37°C, and 0.1 ml of the culture was then inoculated into 2.0 ml of growth media. The inoculum was grown on colonic mucin for the colonic mucin experiments and gastric mucin for all other experiments. Growth of the bacteria was followed by measuring the optical density at 600 nm. After 30 h of growth, the cultures were centrifuged at 10,000 x g for 10 min to remove cells. Mucin carbohydrates were determined
before and after growth by gas-liquid chromatography. Neuraminic acids were assayed colorimetrically by the method of Warren (1959) after hydrolysis for 1 h at 80°C in 0.05 M H$_2$SO$_4$. Ascending gel filtration chromatography was performed using Sepharose 4B and the samples were eluted with 0.1 M NaCl containing 0.01 M sodium phosphate buffer, pH 7.5.

The growth experiments were repeated four times with minor variations. The *Bacteroides* strains were re-isolated onto mucin-containing media twice from the original type cultures that had not previously been grown on mucin media. These were maintained on the medium used by Sperry *et al.* (1977) to which 2 µg of heme per ml and 1.5% agar had been added.

In a separate experiment *Bacteroides fragilis* ATCC 25285 was also grown on gastric mucin (10 mg/ml) that had been isolated from fresh mucosal scrapings. The same cultural and analytical methods were used except that the molecular size on Sepharose 4B was also monitored by the Alcian blue assay at pH 5.8.

Results

Growth on mucins

The growth of the *Bacteroides* strains on colonic mucin, Sigma gastric mucin and glucose is shown in Figure 4.1. Both strains grew better on gastric mucin than colonic mucin. There was negligible growth on the basal medium only. For comparative purposes the bacteria were also grown on 3 mM and 6 mM glucose. Normally at least 11 mM glucose is used in culture medium.

In two experiments the numbers of cells were also counted at the end of the growth experiment using a haemocytometer. Cell counts
Figure 4.1 Growth of *Bacteroides fragilis* strains

(a) ATCC 25285 and (b) ATCC 23745 on gastric mucin at 5 mg/ml (□□□□) and 10 mg/ml (■■■■); on colonic mucin at 10 mg/ml (△△△△) and 20 mg/ml (▲▲▲▲); on glucose at 3 mM (○○○○) and 6 mM (●●●●); with no added carbohydrate (▽▽▽▽).
of $1\times 10^9$ were recorded for both strains growing on gastric and colonic mucins (10 mg/ml). This is approximately one-fifth of the maximum cell density for *Bacteroides fragilis* in broth culture (Frantz *et al.*, 1980).

**Utilisation of mucin sugars**

The mucin sugars found before and after digestion are summarised in Table 4.1. The trace amounts of mannose in the colonic and gastric mucins were degraded by both strains of *Bacteroides*. Most of the gastric neuraminate disappeared but about 75% of the colonic neuraminate was utilised. There was no free neuraminic acid in the culture medium after digestion. Varying amounts of galactose, N-acetylgalactosamine and N-acetylglucosamine were digested but fucose appeared to be conserved. From 8 to 15 mol% of the gastric mucin carbohydrate and 6 to 7 mol% of the colonic mucin carbohydrate was utilised.

The ability of the *Bacteroides* strains to metabolise free neuraminic acid was checked by incorporating 2 mM neuraminic acid (Sigma type VII NeuNAc from human urine) into the basal medium. After 24 hours growth no neuraminic acid was detectable in the culture supernatant of *Bacteroides fragilis* ATCC 25285 but 0.6 mM was left in the supernatant of the *B. fragilis* ATCC 23745 inoculated culture. The neuraminic acid in an uninoculated but incubated control tube was stable over this time period.

**Effect on molecular size of mucins**

Sepharose 4B chromatography of the gastric mucin digests (Fig. 4.2) shows that *B. fragilis* ATCC 25285 could increase the proportion of Sigma gastric mucin that was in the included volume of the gel. When grown on 20 mg per ml mucin for 40 h the high
<table>
<thead>
<tr>
<th></th>
<th>Fuc</th>
<th>Man</th>
<th>Gal</th>
<th>GalNAc</th>
<th>GlcNAc</th>
<th>NeuNAc</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>(a) Undigested mucin</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ATCC 25285</td>
<td>3.1</td>
<td>0.1</td>
<td>10.3</td>
<td>7.0</td>
<td>10.7</td>
<td>0.3</td>
<td>31.5</td>
</tr>
<tr>
<td>ATCC 23745</td>
<td>3.0</td>
<td>0</td>
<td>7.9</td>
<td>6.3</td>
<td>9.6</td>
<td>0.1</td>
<td>26.9</td>
</tr>
<tr>
<td><strong>(b) Undigested mucin</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ATCC 25285</td>
<td>3.4</td>
<td>0.1</td>
<td>7.8</td>
<td>5.5</td>
<td>8.4</td>
<td>1.9</td>
<td>27.1</td>
</tr>
<tr>
<td>ATCC 23745</td>
<td>3.3</td>
<td>0</td>
<td>7.3</td>
<td>4.8</td>
<td>8.3</td>
<td>1.6</td>
<td>25.3</td>
</tr>
<tr>
<td><strong>(c) Undigested mucin</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ATCC 25285</td>
<td>4.1</td>
<td>-</td>
<td>9.6</td>
<td>3.8</td>
<td>9.2</td>
<td>0.7</td>
<td>27.4</td>
</tr>
<tr>
<td>ATCC 23745</td>
<td>3.6</td>
<td>-</td>
<td>4.5</td>
<td>2.1</td>
<td>5.0</td>
<td>0.4</td>
<td>15.6</td>
</tr>
</tbody>
</table>

(a) Utilisation of Sigma gastric mucin by *B. fragilis*.

(b) Utilisation of colonic mucin by *B. fragilis*.

(c) Utilisation of the gastric mucin preparation by *B. fragilis* ATCC 25285.
Figure 4.2  Sigma gastric mucin after digestion by *Bacteroides fragilis*

Molecular size distribution on Sepharose 4B of the PAS reactivity before inoculation (□—□) and after digestion by *B. fragilis* ATCC 25285 (○—○) and ATCC 23745 (△—△).
molecular weight peak was almost totally degraded into the included volume peak. A similar shift was not observed after growth of *B. fragilis* ATCC 23745 on gastric mucin. The colonic mucins (Fig. 4.3) were not detectably altered in molecular size.

**Growth of *B. fragilis* ATCC 25285 on the gastric mucin preparation**

In a separate experiment *B. fragilis* ATCC 25285 grew better on the gastric mucin (10 mg/ml) extracted in the laboratory than on either the Sigma gastric or the colonic mucin preparation (data not shown). Maximum optical density increases of 0.38 and 0.4 O.D. were recorded compared to a maximum of 0.30 O.D. for growth on Sigma gastric mucin at 10 mg per ml. This difference was also apparent in the analysis of the carbohydrates consumed. Nearly 40 mol% of the available sugars were utilised (Table 4.1). These were mainly galactose and N-acetylgalacosamine.

The molecular size of the mucins after digestion (Fig. 4.4) shows that the periodate positive peak has decreased in size but still elutes at the same position as the undigested control (Fig. 4.11). Alcian blue binding material, however, has appeared in the included region of the gel. This material did not have a detectable peak at 260 nm but was not analysed further.

**Discussion**

The results of this section are considered in Chapter Five.
Figure 4.3  Colonic mucin after digestion by *Bacteroides fragilis*

Molecular size distribution on Sepharose 4B of the PAS reactivity before inoculation (□—□) and after digestion by *B. fragilis* ATCC 25285 (○—○) and ATCC 23745 (△—△).
Molecular size distribution on Sepharose 4B of the PAS (\(\triangle\)) and Alcian blue (\(\bullet\)) reactivity remaining after 24h digestion of the gastric mucin preparation by \textit{B. fragilis} ATCC 25285.

A sample of 0.5 ml was applied to the column and the apparent mucin concentration calculated using standard curves of the gastric mucin preparation. Alcian blue reactivity was determined at pH 5.8.
4.2 Degradation of mucins by isolates from the pig colonic mucosa

Introduction

Three attempts to isolate bacteria from the colonic surface of mice yielded only low colony counts and the plates were contaminated by a rapidly spreading *Clostridium tetani*-like organism. Two separate isolations were subsequently made from the pig colonic mucosa and a few of the cultures obtained were used to study the bacterial degradation of mucins.

Trials of different basal media were also carried out which revealed that rumen-fluid had sufficient substrates to support the growth of many bacteria, although the preincubation technique of Dehority and Grubb (1976) can be used to eliminate these. The semi-synthetic Medium 10 (Caldwell and Bryant, 1977) was therefore adopted as a basal medium with the carbohydrates being substituted by mucins. This medium contains small quantities of Trypticase and yeast extract which do not interfere with the analysis of mucin carbohydrates or allow growth in the absence of added energy sources. Allison *et al.* (1979) had demonstrated that Medium 10 could support the growth of $10^9$ bacteria per gram (dry wt) pig caecal contents.

Bacteria were isolated from the same site as mucus was harvested from in the pig colon. It was thought that degradation of mucin might possibly be enhanced if species and site specificity existed.

Isolation of the bacteria

Method

A 5 cm section of colon was ligated immediately after exposure of the gut at the apex of the centripetal and centrifugal coils of a
grain-fed porker pig. The section was cut out and placed in sterile, pre-reduced basal medium (Table 2.1). All air bubbles were excluded by overflowing the flask and sealing it with a rubber bung. Excess medium was allowed to flow out the channel created by a thin wire between the glass and the bung. The wire was removed and the flask transported back to the laboratory at room temperature. Resazurin in the medium rapidly turned colorless after sealing the flask, and isolation procedures were started 25 min after collection.

Inside the anaerobic chamber, a section of the gut mucosa was cut out (1.5 g wet wt after processing) and washed by vigorous agitation in 5 changes of 50 ml basal medium. The secretory surface was then scraped with a sterile scalpel blade to collect the mucus layer without cutting into the underlying mucosa. Ten-fold serial dilutions of the mucoid material were made into basal medium and dispersed by vigorous rotamixing.

The bacteria were isolated by spreading 0.1 ml of the $10^{-3}$ and $10^{-4}$ dilutions onto basal-medium agar plates that contained 2.5 mg per ml of colonic mucin. After 48 h growth, 52 isolated colonies were selected at random and transferred onto other agar plates containing pig colonic mucin. These cultures were grown for 2 days and then used as an inoculum to compare the growth on basal medium agar containing either colonic mucin (2.5 mg/ml), or gastric mucin (2.5 mg/ml), or glucose plus starch and cellobiose (2 mg/ml each), or no added carbohydrate.

A range of different morphological types of bacteria that grew well on mucin, but poorly or not at all on basal medium without added carbohydrates were selected for further study. These were subcultured from single colonies at least five times. The primary
cultures usually contained several bacterial types. The main morphological type that had been found in the primary isolation was selected on subculture.

In addition to the agar plating, the numbers of mucin-degrading bacteria were enumerated in broth culture. Samples of 0.1 ml from the $10^0$ to $10^{-5}$ serial dilutions of the mucosal scraping were inoculated, in duplicate, into broth tubes of basal medium containing 2.5 mg per ml of colonic mucin. The tubes were incubated at 37°C and growth was monitored from the optical density at 660 nm for 42 h.

Results

Counts of bacterial colonies which grew on the colonic mucin agar plates indicated that there were at least $7 \times 10^5$ mucin-degrading bacteria per ml of inoculum. The $10^{-5}$ serial dilution of the inoculum that was grown in colonic mucin broth showed growth only after a lag phase of 24 h (Fig. 4.5). Therefore approximately $10^6$ bacteria, per gram (wet wt) of mucosal tissue, could grow on the colonic mucin media. This number is less than that found by Allison et al. (1979) who isolated bacteria from the caecal wall of pigs using 2 mg per ml Sigma pig gastric mucin in a carbohydrate-depleted rumen-fluid basal medium. They found $10^8$ to $10^9$ bacteria per gram (dry wt) of tissue but the study was done by homogenising the whole caecal wall.

Identification of cultures

A presumptive identification of the isolates was carried out using the procedures outlined in the "Anaerobe Laboratory Manual", 4th Edition, Virginia Polytechnic Institute and State University
Figure 4.5  Growth on colonic mucin of serial dilutions of bacteria from pig colon mucus.

Mucus was obtained from the pig colonic mucosal surface as described in section 4.2. Ten-fold serial dilutions were done into basal medium without added carbohydrate and 0.1 ml of $10^0$ (●●●), $10^{-1}$ (□□□), $10^{-2}$ (△△△), $10^{-3}$ (○○○), $10^{-4}$ (●○○) and $10^{-5}$ (■■■) was inoculated into 2 ml of basal medium containing 2.5 mg/ml colonic mucin.

Duplicate tubes were incubated anaerobically at 37°C. Uninoculated tubes of colonic mucin (▽▽▽) showed no growth.
(Holdeman, L.V., Cato, E.P. and Moore, W.E.C. Eds.). The assistance of R. Menzies, Microbiology Section, Department of Pathology, Green Lane Hospital, Auckland is gratefully acknowledged.

Results

Table 4.2 shows test results relevant to the identification of the organisms. Culture 2 is probably a Bacteroides sp. The fermentation products suggest Bacteroides oralis which is found as normal flora in the intestine of man and animals. Culture 13 shows properties consistent with an identification of a Bacteroides sp. but no species could be assigned. Culture 42 is Clostridium perfringens type A although spore production could not be demonstrated by the heat test. This bacterium is also found as normal flora in the intestine of man and animals.

Utilisation of mucins by isolates from the pig colon

Introduction

Of the ten bacterial cultures isolated from the pig colon and selected for study only three showed substantial colonic mucin-degrading ability when screened by the colorimetric anthrone and neuraminate assays. These cultures were therefore examined in more detail. Their ability to live on Sigma gastric mucin and colonic mucin as energy sources was determined at least four times on different batches of mucins and at different mucins concentrations. The proportion of individual mucin sugars fluctuated up to 20% among different batches of the same mucin, but the relative extent of mucin degradation between experiments was similar. The types of sugar residues cleaved by each culture were also consistent between experiments. Results presented here are typical. The growth curves,
<table>
<thead>
<tr>
<th>Culture</th>
<th>Test</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>Gram stain</td>
<td>Gram negative bacillus of variable size.</td>
</tr>
<tr>
<td></td>
<td>Black pigment</td>
<td>Negative</td>
</tr>
<tr>
<td></td>
<td>Acid from salicin</td>
<td>Negative</td>
</tr>
<tr>
<td></td>
<td>Indole production</td>
<td>Negative</td>
</tr>
<tr>
<td></td>
<td>Aesculin hydrolysis</td>
<td>Positive</td>
</tr>
<tr>
<td></td>
<td>Growth on 20% bile</td>
<td>Inhibited</td>
</tr>
<tr>
<td></td>
<td>Fermentation products</td>
<td>Acetate, isobutyrate, isovalerate succinate.</td>
</tr>
<tr>
<td>13</td>
<td>Gram Stain</td>
<td>Small Gram negative bacillus</td>
</tr>
<tr>
<td></td>
<td>Black pigment</td>
<td>Negative</td>
</tr>
<tr>
<td></td>
<td>Acid from salicin</td>
<td>Negative</td>
</tr>
<tr>
<td></td>
<td>Aesculin hydrolysis</td>
<td>Negative</td>
</tr>
<tr>
<td></td>
<td>Indole production</td>
<td>Negative</td>
</tr>
<tr>
<td></td>
<td>Growth on 20% bile</td>
<td>Inhibited</td>
</tr>
<tr>
<td></td>
<td>Fermentation products</td>
<td>Acetate, minor isovalerate, minor succinate.</td>
</tr>
<tr>
<td>42</td>
<td>Gram stain</td>
<td>Large Gram positive bacillus</td>
</tr>
<tr>
<td></td>
<td>Colonial appearance</td>
<td>Non-haemolytic, grey, mucoid, large</td>
</tr>
<tr>
<td></td>
<td>Gas produced</td>
<td>Abundant gas</td>
</tr>
<tr>
<td></td>
<td>Indole production</td>
<td>Negative</td>
</tr>
<tr>
<td></td>
<td>Lactose</td>
<td>Positive</td>
</tr>
<tr>
<td></td>
<td>Sucrose</td>
<td>Positive</td>
</tr>
<tr>
<td></td>
<td>Lecithinase</td>
<td>Positive</td>
</tr>
<tr>
<td></td>
<td>Motility</td>
<td>Negative</td>
</tr>
<tr>
<td></td>
<td>Fermentation products</td>
<td>Butyrate, acetate, propionate (minor)</td>
</tr>
<tr>
<td></td>
<td>Clostridium perfringens</td>
<td>Positive</td>
</tr>
<tr>
<td></td>
<td>Type A, antitoxin</td>
<td></td>
</tr>
</tbody>
</table>
Sepharose columns and mucin composition data are derived from the same experiment using the same batch of each mucin.

This was the only experiment which tested the bacterial degradation of gastric mucin prepared from fresh mucosal scrapings as opposed to the Sigma gastric mucin.

Method

Bacteriological procedures

The cultures were maintained on agar slopes of basal medium containing at least 10 mg per ml of Sigma gastric mucin. An inoculum was prepared by transferring a loopful of culture from a freshly grown agar slope to 3 ml of basal medium broth containing either Sigma gastric or colonic mucin at 10 mg per ml. The optical density of the cultures was monitored at 660 nm and 0.4 ml of log phase culture (6–12 h growth) was used to inoculate 3 ml of the appropriate medium. Bacteria grown on colonic mucin were used to inoculate the colonic mucin tubes. All other tubes were inoculated with bacteria grown on Sigma gastric mucin.

The growth of the cultures was monitored by following the optical density at 660 nm for 24 h. Cultures were then centrifuged at 10,000 x g for 10 min to remove bacterial cells, and the supernatants were stored at -10°C for later analysis. Bacterial cells from every tube were Gram stained to check for contamination.

The mucins were examined by Sepharose 4B chromatography before and after digestion by the bacteria. Fractions were collected and monitored by the PAS and Alcian blue (pH 5.8) assays. These were semi-quantified by including a standard curve of the appropriate undigested mucin with every column analysis.
The mucin sugars remaining in the medium were analysed by gas-liquid chromatography of the trimethylsilyl derivatives. Results were quantified by cutting out and weighing the area under each peak.

Results and discussion

Growth of the cultures on mucins

All the cultures showed a greater optical density increase when growing on basal medium plus mucins than on basal medium alone (Fig. 4.6). Peak growth was reached within 24 h. Incubation of the bacteria beyond 24 h may have released intracellular glycosidases.

Culture 2 reached a higher optical density while growing on Sigma gastric mucin than while growing on colonic mucin. Culture 13 grew very well on the colonic and gastric mucin preparations but growth on the Sigma mucin stopped after approximately 6 h. The cause is unknown but lysis of the cells was observed more than once.

Culture 42 grew well on the gastric mucins and colonic mucins. This bacterium is a long Gram-positive rod and shows less turbidity than culture 2 and 13 at full growth. The cells clumped and fell rapidly out of solution causing fluctuations in the optical density readings.

Utilisation of the mucin carbohydrates

The analyses of the carbohydrates in the media before and after digestion are detailed in Tables 4.3, 4.4 and 4.5 and are also presented diagrammatically in Figures 4.7, 4.8 and 4.9.

All the cultures cleaved more than 50% of the Sigma gastric mucin carbohydrates from the glycoprotein but only culture 13 was effective in cleaving more than half the residues from the gastric
Figure 4.6  Growth of cultures from the pig colon on mucins

Basal medium containing 1% (w/v) mucin was inoculated with (a) culture 2, (b) culture 13 and (c) culture 42 as detailed in Section 4.2

The cultures were grown on colonic mucin (□—□), Sigma gastric mucin (△—△), gastric mucin prepared from mucosal scrapings (○—○), and on basal medium without added carbohydrate (▽—▽). Net optical density increases have been shown except for the tubes of cultures 13 and 42 growing in basal medium only where slight decreases were recorded.
### Table 4.3 Utilisation of Sigma gastric mucin by isolates from pig colon

Mucin carbohydrate remaining after digestion (mM)\(^a\)

<table>
<thead>
<tr>
<th></th>
<th>Fuc</th>
<th>Gal</th>
<th>GalNac</th>
<th>GlcNac</th>
<th>NeuNac</th>
<th>Total</th>
<th>b,c,d</th>
</tr>
</thead>
<tbody>
<tr>
<td>Undigested mucin</td>
<td>3.1</td>
<td>10.4</td>
<td>10.7</td>
<td>13.0</td>
<td>0.4</td>
<td>37.6</td>
<td></td>
</tr>
<tr>
<td>Culture 2</td>
<td>2.6/1.9</td>
<td>3.0</td>
<td>5.8</td>
<td>3.2</td>
<td>0.4/0.5</td>
<td>15.0</td>
<td></td>
</tr>
<tr>
<td>Culture 13</td>
<td>3.0/1.5</td>
<td>6.5/1.9</td>
<td>7.8</td>
<td>3.2/2.1</td>
<td>0.0</td>
<td>20.5</td>
<td></td>
</tr>
<tr>
<td>Culture 42</td>
<td>2.8/2.5</td>
<td>6.8/1.1</td>
<td>4.5</td>
<td>3.0/0.6</td>
<td>0.1</td>
<td>9.6</td>
<td></td>
</tr>
</tbody>
</table>

(a) The carbohydrates were determined by gas-liquid chromatography. Neuraminic acids were determined by the method of Warren (1959).

(b) Samples were in duplicate. Analysis of individual sugars agreed to within 15%.

(c) Mucin was added at 10 mg/ml (freeze dried weight).

(d) Total carbohydrate/Free carbohydrate.
Table 4.4  Utilisation of the gastric mucin preparation by isolates from pig colon

Mucin carbohydrates remaining after digestion (mM)\(^{a}\)

<table>
<thead>
<tr>
<th></th>
<th>Fuc</th>
<th>Gal</th>
<th>GalNac</th>
<th>GlcNac</th>
<th>NeuNac</th>
<th>Total (^{b,c,d})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Undigested mucin</td>
<td>4.1</td>
<td>9.6</td>
<td>3.8</td>
<td>9.2</td>
<td>0.7</td>
<td>27.4</td>
</tr>
<tr>
<td>Culture 2</td>
<td>3.5/</td>
<td>4.8</td>
<td>3.6</td>
<td>6.0</td>
<td>0.5/</td>
<td>18.4</td>
</tr>
<tr>
<td></td>
<td>1.8</td>
<td></td>
<td></td>
<td></td>
<td>0.4</td>
<td></td>
</tr>
<tr>
<td>Culture 13</td>
<td>1.8/</td>
<td>1.2</td>
<td>0.1</td>
<td>1.1</td>
<td>0.1</td>
<td>4.3</td>
</tr>
<tr>
<td></td>
<td>1.5</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Culture 42</td>
<td>2.3/</td>
<td>6.2/</td>
<td>2.6</td>
<td>6.6</td>
<td>0.1</td>
<td>17.8</td>
</tr>
<tr>
<td></td>
<td>2.0</td>
<td>0.3</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\(^{a}\) The carbohydrates were determined by gas-liquid chromatography. Neuraminic acids were determined by the method of Warren (1959).

\(^{b}\) Samples were in duplicate. Analysis of individual sugars agreed to within 15%.

\(^{c}\) Mucin was added at 10 mg/ml (freeze dried weight).

\(^{d}\) Total carbohydrate/Free carbohydrate.
Table 4.5  Utilisation of colonic mucin by isolates from the pig colon

<table>
<thead>
<tr>
<th></th>
<th>Fuc</th>
<th>Gal</th>
<th>GalNAc</th>
<th>GlcNAc</th>
<th>NeuNAc</th>
<th>Total b,c,d</th>
</tr>
</thead>
<tbody>
<tr>
<td>Undigested mucin</td>
<td>5.0</td>
<td>11.0</td>
<td>4.6</td>
<td>10.5</td>
<td>1.5</td>
<td>32.6</td>
</tr>
<tr>
<td>Culture 2</td>
<td>4.1/</td>
<td>5.6</td>
<td>4.6</td>
<td>6.7</td>
<td>1.5/</td>
<td>22.5</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td></td>
<td></td>
<td>0.8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Culture 13</td>
<td>4.5/</td>
<td>4.9/</td>
<td>4.1</td>
<td>5.3/</td>
<td>0.6</td>
<td>18.1</td>
</tr>
<tr>
<td></td>
<td>5.0</td>
<td>3.6</td>
<td></td>
<td>4.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Culture 42</td>
<td>4.6</td>
<td>6.8</td>
<td>4.8</td>
<td>7.9</td>
<td>1.5</td>
<td>25.6</td>
</tr>
</tbody>
</table>

(a)  The carbohydrates were determined by gas-liquid chromatography. Neuraminic acids were determined by the method of Warren (1959).

(b)  Samples were in duplicate. Analysis of individual sugars agreed to within 15%.

(c)  Mucin was added at 10 mg/ml (freeze dried weight).

(d)  Total carbohydrate/Free carbohydrate.
Figure 4.7  Utilisation of Sigma gastric mucin by isolates from pig colon

Diagrammatic summary of Table 4.3
Figure 4.8 Utilisation of the gastric mucin preparation by isolates from pig colon

Diagrammatic summary of Table 4.4
Figure 4.9  Utilisation of colonic mucin by isolates from pig colon

Diagrammatic summary of Table 4.5
or colonic mucin preparations. This culture utilised 15 and 23 mM carbohydrate from the colonic and gastric mucin preparations respectively but cultures 2 and 42 only removed 7-10 mM carbohydrate from the media containing these mucins. The media contained between 27 and 37 mM carbohydrate in total.

Galactose and N-acetylgalcosamine were used by all the cultures with fucose and N-acetylgalactosamine being utilised to a lesser extent. N-Acetyleneuraminsic acid was utilised by culture 13 but cultures 2 and 42 also showed neuraminidase activity. The gastric mucin neuraminic acid content appeared more susceptible to hydrolysis than the colonic neuraminate. No more than 60% of the colonic mucin neuraminic acids were released whereas almost all the gastric mucin neuraminic acid content was hydrolysed.

**Sepharose chromatography of the mucin digests**

The individual chromatograph columns are shown in Figures 4.10 to 4.21. A summary of these is presented in Figure 4.22.

The Sigma gastric mucin shows a consistent pattern in which the PAS reactive peak was substantially reduced in size leaving the Alcian blue peak largely unchanged. The gastric mucin preparation also showed a major loss of the PAS-reactive peak but Alcian blue reactivity was modified by cultures 2 and 13. Culture 42 did not affect the Alcian blue reactivity of gastric mucin preparation to the same degree as the other cultures.

Colonic mucin was not detectably reduced in molecular size by any of the cultures. The PAS and Alcian blue reactivities had parallel curves after digestion which peaked in the same position as the undigested preparation. Culture 13 showed the most reduction in size
of the colonic mucin peaks with the PAS reactivity decreasing more
than the Alcian blue reactivity.
Figure 4.10  Undigested Sigma gastric mucin

Molecular size distribution on Sepharose 4B of the PAS (△—△) and Alcian blue (●—●) reactivities of Sigma gastric mucin before bacterial digestion.

A sample of 0.5 ml was applied to the column and the apparent mucin concentration calculated from a standard curve of the Sigma gastric mucin. Alcian blue reactivity was determined at pH 5.8.
Molecular size distribution on Sepharose 4B of the PAS (△–△) and Alcian blue (●–●) reactivities of Sigma gastric mucin before bacterial digestion.

A sample of 0.5 ml was applied to the column and the apparent mucin concentration calculated from a standard curve of the gastric mucin preparation. Alcian blue reactivity was determined at pH 5.8.
Figure 4.12  Undigested colonic mucin

Molecular size distribution on Sepharose 4B of the PAS (□□) and Alcian blue (▲▲) reactivities of colonic mucin before bacterial digestion.

A sample of 0.5ml was applied to the column and the apparent mucin concentration calculated from a standard curve of the gastric mucin preparation. Alcian blue reactivity was determined at pH 5.8.
Figure 4.13  Sigma gastric mucin after digestion by culture 2.

Molecular size distribution on Sepharose 4B of the PAS (△—△) and Alcian blue (●—●) reactivities of Sigma gastric mucin after 24h digestion by culture 2.

A sample of 0.5 ml was applied to the column and the apparent mucin concentration was calculated from standard curves of the Sigma gastric mucin. Alcian blue reactivity was determined at pH 5.8.
Molecular size distribution on Sepharose 4B of the PAS (▽▽) and Alcian blue (●●) reactivities of the gastric mucin preparation after 24h digestion by culture 2.

A sample of 0.5 ml was applied to the column and the apparent mucin concentration calculated from standard curves of the gastric mucin preparation. Alcian blue reactivity was determined at pH 5.8.
Figure 4.15  Colonic mucin after digestion by culture 2.

Molecular size distribution on Sepharose 4B of the PAS (□—□) and Alcian blue (▲—▲) reactivity remaining after 24h digestion of colonic mucin by culture 2.

A sample of 0.5 ml was applied to the column and the apparent mucin concentration calculated from standard curves of colonic mucin. Alcian blue reactivity was determined at pH 5.8.
Figure 4.16  *Sigma* gastric mucin after digestion by culture 13.

Molecular size distribution on Sepharose 4B of the PAS (Δ—Δ) and Alcian blue (●—●) reactivities of *Sigma* gastric mucin after digestion by culture 13.

A sample of 0.5 was applied to the column and the apparent mucin concentration calculated from standard curves of the *Sigma* gastric mucin. Alcian blue reactivities were determined at pH 5.8.
Figure 4.17  Gastric mucin preparation after digestion by culture 13.

Molecular size distribution on Sepharose 4B of the PAS (▽-▽) and Alcian blue (●-●) reactivities remaining after 24h digestion of the gastric mucin preparation by culture 13.

A sample of 0.5 ml was applied to the column and the apparent mucin concentration calculated from standard curves of the gastric mucin. Alcian blue reactivity was determined at pH 5.8.
Figure 4.18  Colonic mucin after digestion by culture 13

Molecular size distribution on Sepharose 4B of the PAS (□□□) and Alcian blue (▲▲▲) reactivities of colonic mucin after 24 h digestion by culture 13.

A sample of 0.5 ml was applied to the column and the apparent mucin concentration calculated from standard curves of the colonic mucin. The Alcian blue reactivity was determined at pH 5.8.
Figure 4.19  Sigma gastric mucin after digestion by culture 42.

Molecular size distribution on Sepharose 4B of the PAS (△—△) and Alcian blue reactivity (○—○) remaining after 24h digestion of Sigma gastric mucin by culture 42.

A sample of 0.5 ml was applied to the column and the apparent mucin concentration calculated using standard curves of Sigma gastric mucin. Alcian blue reactivity was assayed at pH 5.8.
Figure 4.20  Gastric mucin preparation after digestion by culture 42

Molecular size distribution on Sepharose 4B of the PAS (▼▼) and Alcian blue (●●) reactivities of the gastric mucin preparation after 24h digestion by culture 42.

A sample of 0.5 ml was applied to the column and the apparent mucin concentration calculated from standard curves of the gastric mucin preparation. Alcian blue reactivity was determined at pH 5.8. The chromatograph was run three times and always showed major separation of the Alcian blue and PAS reactivities.
Figure 4.21 Colonic mucin after digestion by culture 42

Molecular size distribution on Sepharose 4B of the PAS (□—□) and Alcian blue (▲—▲) reactivity remaining after 24h digestion by culture 42.

A sample of 0.5 ml was applied to the column and the apparent mucin concentration calculated from standard curves of colonic mucin.
SUMMARY : BACTERIAL DIGESTS ON 4B

SIGMA GASTRIC GASTRIC COLONIC

Culture 2

Culture 13

Culture 42

FRACTION NUMBER
4.3 Degradation of mucins by rat faecal-pellet flora

Introduction

Cultures 2, 42 and 13 did not degrade the colonic mucin to the same extent as the gastric mucin. These isolated, pure cultures may lack all the enzymes necessary to degrade colonic mucin completely. If extra enzymes are required, then bacteria producing them are probably present in the colon. The faecal flora should therefore be capable of the rapid breakdown of both colonic and gastric mucins.

Method

A faecal pellet was collected from an adult Wistar rat immediately after defecation. It was rapidly placed in 2 ml of pre-reduced basal medium and kept anaerobic with a continuous flow of oxygen-free carbon dioxide gas. The faecal pellet was disintegrated by agitation with a sterile glass rod and heavy particles in the suspension were allowed to settle out for approximately one minute. A 0.05 ml inoculum was pipetted into tubes of basal media (3 ml) containing either gastric mucin (5 mg/ml), or colonic mucin (10 mg/ml), or glucose (6 mg/ml), or no added carbohydrate. The tubes were incubated at 37°C and the increase in optical density was monitored at 600 nm. Samples of 0.25 ml were taken at 12 h intervals from one set of the duplicate tubes. Bacterial cells were removed by centrifugation at 10,000 x g for 10 min and the supernatant was stored at -10°C for later analysis. Each sample was run on a Sepharose 4B column. Fractions of 2 ml were collected and monitored by the PAS reaction (Mantle and Allen, 1978).

The basic experiment was done twice. In the first experiment a supernatant sample from the colonic mucin tubes was used to follow
the loss of fucose and hexose sugars by the anthrone reaction. In
the second experiment 0.05 ml of the 36 h inoculated colonic mucin
tube was reinoculated into a further 3 ml of fresh colonic mucin.
This tube was then monitored for a further 24 h to examine the
effects of preinduction on the rate of colonic mucin degradation.

Results and discussion

The Sepharose data (Figs. 4.24 and 4.25) and the optical
density growth curves (Table 4.6) indicate that the gastric mucin
was fermented faster than the colonic mucin. The bulk of the gastric
mucin was fermented between 12 and 24 h after inoculation. Most of
the colonic mucin was fermented between 24 and 36 h incubation time.
When a sample from the colonic mucin tube was used to inoculate a new
tube the mucin was completely fermented in 24 h. The difference in
lag phase is therefore probably one of bacterial induction rather
than of intrinsic resistance to degradation.

Gastric and colonic mucins have a very similar carbohydrate
composition apart from their neuraminate contents (see Table 3.5).
The same tube was used to inoculate all the media in any one
experiment and therefore each should receive the same inoculum.
Variyam and Hoskins (1981) found that $10^8$ bacteria per gram (dry wt)
of faeces could produce extracellular neuraminidases suggesting it
is unlikely that the rate of neuraminic acid removal limits colonic
mucin degradation. Therefore, more enzymes are probably required to
degrade colonic mucin than gastric mucin.

The Alcian blue colorimetric reaction shows that there are
other differences between the mucins. The major PAS reactive peak
of Sigma gastric mucin does not bind Alcian blue (Fig. 3.8) but the
colonic mucin reacts readily with this dye (Fig. 3.6). As Alcian
blue reactivity indicates the presence of sulphate residues (Chapter Three) a sulphotase could be the rate limiting factor in colonic mucin degradation.

It is notable that the PAS reactive peak of the digested mucins did not shift into the included volume of the Sepharose gel. This is in contrast to the results obtained with single cultures (Figs. 4.13 - 4.21) where lower-molecular-weight PAS positive peaks were produced. A smaller molecular size was also produced when Variyam and Hoskins (1981) treated gastric mucin with faecal culture supernatant. The peptide backbone of the mucin molecule is normally protected from proteases by the close packing of carbohydrate side-chains. If the partially degraded molecule was susceptible to enzymatic cleavage of the peptide backbone the molecular size could decrease very rapidly with the fragments being utilised by bacteria.

An anomaly was also apparent between the approximately 50% of PAS reactivity that had gone from the colonic mucin tubes after 24 h digestion and the 80% of the anthrone reactive sugars that still remained. This could be due to the fact that the anthrone reaction detects free fucose whereas the PAS reaction does not (Section 3.5). Fucose is in a terminal position on the carbohydrate chains and hydrolysis is probably a prerequisite to further mucin degradation.

Greater than 50% of the PAS reaction of colonic mucin was due to bound fucose (Section 3.9) but fucose was not readily utilised by the pure cultures (Figs. 4.7, 4.8, 4.9). Therefore the anomaly between the PAS and anthrone-detected degradation of the colonic mucin may have been due to fucose being cleaved faster than it was utilised.
Figure 4.24 Degradation of Sigma gastric mucin by rat faecal flora

The molecular size on Sepharose '4B of the PAS reactive mucins was determined after 0h ([—□—□]), 12h ([—●—●]) and 24h ([△—△]) incubation.

Rat faecal flora was obtained as described in section 4.3 and 50 μl was inoculated into tubes containing 3 ml of basal media plus 10 mg/ml Sigma gastric mucin. A sample of 0.25 ml of culture supernatant was applied to the column and the PAS reactivity of 1.0 ml in each fraction was determined by the method of Mantle and Allen (1978).
Figure 4.25  Degradation of colonic mucin by complete rat faecal flora

The molecular size on Sepharose 4B of the PAS reactive mucins was determined after 0h (□□□□□) and 24h (■■■■■) incubation. A sample of the 24h culture was reinoculated into a fresh colonic mucin tube and the PAS reactivity determined after 12h (▽▽▽▽▽) incubation. No PAS reactivity was present after 36h in the original tubes or after 24h in the reinoculated tube.

Rat faecal flora was obtained as described in section 4.3 and 50μl was inoculated into 3ml of basal media plus 10mg/ml colonic mucin. A sample of 0.25ml of culture supernatant was applied to the column and the PAS reactivity of 1.0ml in each fraction was determined by the method of Mantle & Allen (1978).
Table 4.6  Growth of rat faecal flora on mucins

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Concentration (mg/ml)</th>
<th>Increase in OD at 600 nm</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0h</td>
<td>3</td>
</tr>
<tr>
<td>No carbohydrate</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Colonic mucin</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td>Sigma gastric mucin</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>Glucose</td>
<td>6</td>
<td>0</td>
</tr>
</tbody>
</table>

Table 4.7  Utilisation of anthrone-reactive sugars of colonic mucin by rat faecal flora

<table>
<thead>
<tr>
<th>Incubation time (hours)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
</tr>
</tbody>
</table>

Percentage of anthrone reactive sugars remaining

<table>
<thead>
<tr>
<th></th>
<th>100%</th>
<th>95%</th>
<th>80%</th>
<th>3%</th>
</tr>
</thead>
</table>

CHAPTER FIVE

GENERAL DISCUSSION

The main objective of this thesis was to determine the potential of mucus glycoprotein to act as one of the energy sources for the large numbers of bacteria growing in the colon. Other investigators in this field have used commercially available pig gastric mucin (Wiel-Korstanje et al., 1975; Allison et al., 1979; Miller et al., 1981; Hoskins et al., 1981). The investigations in this thesis have utilised not only commercial pig gastric mucin, but also mucins prepared from fresh mucosal scrapings of the pig stomach and colon. Methods had to be developed to extract gram quantities of these mucins in order to provide sufficient substrate for bacteriological use. The extraction steps also required close monitoring to ensure that a high yield of glycoprotein was obtained without enzymic digestion of the carbohydrate side-chains. Chemical and molecular size analysis of the products showed that the mucins obtained were similar to those extracted by other workers from the same sources.

The mucins were then used in three types of bacterial experiment. Firstly colon mucin was incorporated in basal media as a selective substrate to isolate bacteria from the mucosal surface of the pig colon. These bacteria extensively degraded mucins. Secondly type cultures of Bacteroides fragilis were analysed for their ability to degrade mucins. In the third set of experiments the mucin degradation by the isolated pure cultures, and the type cultures above, was compared to mucin degradation by a complete spectrum of flora from rat faeces.
All these experiments showed that the colonic mucin was more resistant to bacterial attack than the gastric mucins. The composition of the mucin preparations, however, was similar and did not readily suggest an explanation for this result. Colorimetric analysis of the mucins using histochemical stains did reveal differences between the preparations. The molecular basis of these stains has been more thoroughly investigated. The simplest hypothesis that might explain these results is that certain of the sulphate groups on mucins render them more resistant to bacterial degradation. These results, and the evidence for and against the hypothesis, will be discussed in more detail. Implications of the differences between bacterial degradation by luminal and mucosal bacteria will also be considered.
5.1 Differences between the gastric and colonic mucin preparations

The measurements in Table 3.5 show that the gastric and colonic mucin preparations have similar carbohydrate compositions. Only the neuraminate contents varied significantly being much higher in the colonic mucin. These results are very similar to those reported by Starkey et al., (1974) and Marshall et al., (1978), except that the sulphate content of both preparations in this thesis was less than half that found in the preparations of these other workers.

Sigma gastric mucin had a higher proportion of N-acetylgalactosamine than the other preparations. This could be the result of it possessing a higher blood group A titre, with more chains having at least two N-acetylgalactosamine residues instead of one. Alternatively the average carbohydrate chain length of the Sigma mucin could be shorter, perhaps as a result of bacterial degradation during processing (Appendix 4).

Although the chemical compositions of the gastric and colonic mucin preparations used in this thesis were similar, major differences were noted between them in their reactivity with Alcian blue, PAS, and precipitation by acriflavine and cetyl pyridinium chloride.

Neuraminidase digestion and low pH did not significantly affect the binding of Alcian blue to the mucins. Therefore the Alcian blue binding capacity could be expected to be quantitatively related to sulphate content. This was not confirmed by comparing the sulphate content of the mucins with their Alcian blue reactivity. Despite having similar sulphate (1.0-1.3% w/w) the colonic mucin
preparation gave twice the response (w/w) in the Alcian blue assay than either the Sigma gastric mucin or mucin prepared from fresh mucosal scrapings. Sepharose chromatography, hyaluronidase digestion, DNAase digestion and cellulose acetate electrophoresis all indicated that the Alcian blue reactivity of the gastric and colonic mucin preparations was solely due to acidic mucins. Therefore the discrepancy suggests that Alcian blue does not quantitatively bind to all of the sulphate esters in these mucins.

This was further supported by an investigation of the gastric mucin preparation. The Sepharose chromatography of this gastric mucin preparation suggested that it contained at least two mucin types that varied in their reactivities towards Alcian blue and the PAS reaction. Cetyl pyridinium chloride precipitation was used in an attempt to precipitate the acidic mucin fraction so that the composition and reactivities of the acidic and neutral mucins could be compared. The precipitated mucin was found to contain less than half the dry weight but had three-quarters of the Alcian blue-binding capacity of the combined precipitated and soluble fractions (Section 3.10). However, both these fractions had a sulphate content of 1.0-1.1% (w/w).

Other differences were noted between the mucin preparations. Acriflavin precipitates mucins with ester sulphates (Inoue, 1965). The colonic mucin gave over twice the turbidity (w/w) of the gastric mucins when treated with acriflavin (Section 3.11) It was also found that twice as much CPC (w/w) was required to precipitate the colonic mucin preparations compared to the gastric mucins.
Therefore the results again suggest that only some sulphate esters are able to bind the positive charges on the Alcian blue, CPC and acriflavín molecules.

It is desirable that the histochemical and biochemical studies give comparable results but many discrepancies have been noted by other workers. For example, histochemical staining of human stomach and small intestinal cells show that predominately fucomucins and sialomucins are produced (Filipe, 1979). However, the biochemical preparations of human gastric mucin (Schrager et al., 1971) and human intestinal mucin (Wesley et al., 1982) show the presence of 1-7% (w/w) sulphate ester. These differences are postulated to arise from salivary and oesophageal contamination of the gastric mucin (Filipe, 1979). However, radioactive sulphate uptake by the human gastric epithelium and incorporation into mucins has been demonstrated in vitro (Filipe, 1979).
5.2 A possible mechanism for the failure of histochemical analysis to quantitatively detect mucin sulphate esters

Interpretation of the histochemical detection of mucin sulphates has been largely inferred from studies on polymers of known composition such as chondroitin sulphate because biochemical preparations of pure mucins, with known structures, have not been available. Mucins are not linear molecules of repeating disaccarides like the glycosaminoglycans but are long flexible molecules of a "bottle brush" nature. At low ionic strength these molecules are highly expanded and at a concentration of 30–40 mg per ml can completely fill the solution (Allen, 1978).

It has been suggested that the ability of mucins to selectively exclude molecules is an integral part of their function (Edwards, 1978). Alcian blue, acriflavin and CPC have positively charged amine groups on largely hydrophobic molecules. Their molecular formulae indicate that they will form micelle-like structures and it was noted that these do not readily pass through a dialysis membrane (personal observations). Therefore hydrophobic exclusion or steric hindrance may prevent them from binding to sulphate or neuraminate residues that are located in the interior of the mucin molecule near the peptide backbone. This effect would be accentuated if the mucin gel was in a collapsed state due to fixation prior to histochemical staining.

An alternative explanation was suggested by Spicer (1960) when trying to explain why apparently sulphated colonic epithelial mucins failed to stain with Alcian blue. He suggested that
equatorially directed sulphate residues would be less sterically hindered that axial ones and therefore would interact more easily with the stain.

These suggestions are speculative but there is indirect evidence from other workers that sulphate esters can occur in more than one position on mucin carbohydrate chains. Slomiany and Meyer (1972) found that pig gastric glycopeptides with more than one sulphate residue per chain had lower blood group A reactivity. They suggested that this was because a sulphate residue was located near the non-reducing terminus. Liau et al., (1982) isolated hexosamine monosulphate and disulphate as well as galactose sulphate from rat gastric mucin.

In this thesis (Section 3.9) it was noted that Alcian blue reactivity of the mucins was lost by acid hydrolysis at a faster rate than would be expected if the dye was bound to internally located primary sulphate esters. When gastric mucin was fractionated by cetyl pyridinium chloride it was also found that the precipitated fraction had a high Alcian blue response (w/w) and low PAS reactivity (w/w) compared to the soluble fractions which showed the opposite effect. This result would not be predicted on the basis of the carbohydrate or sulphate contents as both mucins are similar. Because the PAS-reactive carbohydrates are located mainly at the non-reducing terminal (Section 3.9) internally located sulphate residues should not affect the PAS reaction but externally located sulphate residues might.
Further research is required to conclusively prove or disprove whether some sulphate esters are not reactive to the histochemical stains such as Alcian blue.
5.3 Relationship between the moles of N-acetylgalactosamine and sulphate to the length of the mucin oligosaccharide chains

Bacterial degradation of mucins is thought to proceed by the sequential cleavage of sugars from the non-reducing end of the carbohydrate chains (Chapter 1). N-acetylgalactosamine residues have only been found as either blood group determinants at the non-reducing terminal, or as the linkage sugar to the protein backbone. (Figs. 1.1, 1.2 and 1.3) Therefore in a partially degraded glycoprotein the residual N-acetylgalactosamine is likely to represent the fraction that occurs as linkage sugar. This is even more likely if N-acetylgalactosamine cleavage can be demonstrated to have occurred as hexosaminidases, which cleave non-reducing terminal residues, show a low degree of specificity towards the type of hexosamine that is hydrolysed (Kobata, 1978). The biochemical analyses of the mucins (Tables 4.3, 4.4 and 4.5) can therefore be used to estimate the proportion of galactosamine that occurs as the linkage sugar and, from that, the average length and degree of sulphation of the carbohydrate chains.

None of the cultures from the pig colon hydrolysed more than 11% of the galactosamine residues from pig colonic mucin despite cleaving up to 89% of the glucosamine residues. Therefore most N-acetylgalactosamine residues are likely to be linkage sugars and the minimum average length of the oligosaccharide chains is probably 7. Similarly, in the digestions of the gastric mucin prepared from fresh mucosal scrapings, Culture 13 appeared to completely hydrolyse the mucin but Cultures 2 and 42 cleaved no more than 30% of the N-acetylgalactosamine residues. If all the
residues in the undigested mucin are linkage sugars the minimum average chain length is 7. If only half the residues are linkage sugars the minimum average chain length is 14.

The Sigma gastric mucin preparation was different, however. The cellulose acetate electrophoresis indicated that this mucin contained more of the neutral mucin species than the gastric mucin prepared from fresh mucosal scrapings. It also had a much higher N-acetylgalactosamine content, of which 27 to 58% was digested by the cultures isolated from the pig colon. If only half the galactosamine residues were located at the reducing terminal then the minimum average chain length is 7. If all the residues are located at the non-reducing terminal then the minimum average chain length is 3.5.

These calculations, however, assume that only one mucin species is present. The gastric mucins have been shown to be mixtures of at least two mucins (Section 3.2) each of which could have a different average chain length. In addition calculations of the average number of sugar residues per oligosaccharide chain do not reveal anything about the range of lengths present. Clamp and Gough (1979) found that, although the average number of sugars per oligosaccharide chain in fetal meconium was 6 to 7, some chains were up to triple that length.

The above assumption, that there is mainly one N-acetylgalactosamine residue per chain, has been made on the evidence of the
bacterial digestion data. Slomiany et al., (1972) assumed that there were at least two N-acethylgalactosamine residues per chain in pig gastric mucus because the glycoproteins had blood group A activity. Marshall and Allen (1978) showed that pig colonic mucus also had blood group A activity and that this was increased by neuraminidase treatment. However, the bacteria digestion evidence presented here, and the data of Wesley et al., (1982), who took account of blood group secretor status, suggest that less than half the chains of the pig gastric and colonic mucin have terminal N-acetylgalactosamine residues. Therefore the average chain lengths of 14 and 18 calculated by Slomiany et al., (1972) could be a considerable over-estimate of the actual number.

Using the same assumptions that were made to calculate the average chain length it is also possible to calculate the average number of sulphate residues per chain. For the colonic and gastric mucin preparations there is an average of 0.29 moles of sulphate per mole of N-acetylgalactosamine. This is equivalent to a minimum of one sulphate residue per 3.5 chains if the average chain length is 7. Wesley et al., (1982) have shown that there is a positive correlation of sulphate content with carbohydrate chain length. They found an average of 0.45 moles of sulphate per N-acetylgalactosamine residue in human small intestinal mucin. However, the range between individual samples was 0.17 to 0.93 moles of sulphate per galactosamine residue. Marshall and Allen (1978) found that their preparation of pig colonic mucin had an average of 0.69 moles of sulphate per mole of N-acetylgalactosamine.
The range of values given above may be due to random variation between samples or possibly differences in the type of mucus collected. Colonic mucus collected for this thesis was from the middle of the large intestine, whereas Marshall and Allen (1978) collected mucus from the terminal end near the anus. The low number of sulphate residues per N-acetylgalactosamine has also not yet been accounted for by any quantitative sequencing analysis of isolated carbohydrate chains. A possible model to fit the available data, however, could be that most sulphate residues are located on the longer carbohydrate chains. There could also be chains with more than one sulphate residue as suggested by the data of Slomiany et al., (1972) and Liau et al., (1982).
5.4 Resistance of colonic mucin to degradation

The summary of the Sepharose chromatography results in Figure 4.22 illustrates the consistent finding that pig colonic mucin was more resistant to degradation by isolates from the pig colon than either of the gastric mucin preparations. This was also shown by the *B. fragilis* cultures and colonic mucin was not degraded as rapidly as gastric mucin by rat faecal flora. The cause of this difference is not readily apparent from the analysis of the mucins.

Removal of the outer chain sugars, neuraminic acid and fucose, did not appear to be a limiting factor. Both these residues can sterically inhibit glycosidase action (Kobata, 1978) but all the cultures showed the ability to cleave these sugars from the gastric mucins. Therefore it must be assumed that colonic mucin contains different bonds from gastric mucin and that the cultures were not able to readily hydrolyse such bonds.

An indication that there were differences between the mucins was revealed in their reactivity to cationic dyes such as Alcian blue. The Sepharose chromatography data showed that Alcian blue reactivity of the gastric and colonic mucins was conserved more than PAS staining during bacterial degradation but the results of the Sigma mucin were obscured by hyaluronic acid. Some of the Alcian blue reactivity, however, was lost from the gastric and colonic mucin preparations digested by Cultures 2 and 13, but much of it still remained. Therefore the results suggest that Alcian blue reactive sulphate esters are associated with the inhibition of mucin degradation.
Detailed studies on mucin degradation by a few pure cultures however is not statistically good evidence that colonic mucin is more resistant to degradation than gastric mucin in vivo. An attempt was therefore made to investigate degradation by mixed luminal flora. When the mucins were inoculated with rat faeces the colonic mucin took up to 12 h longer to be degraded than the Sigma gastric mucin. This implied that there may also be differences between the degradation of gastric and colonic mucin in the large intestine. An indication of the cause of such differences was obtained by reinoculation of bacteria from the digested colonic mucin tube into fresh colonic mucin. This showed that degradation could proceed rapidly when the right bacteria had been selected for.

The simplest explanation for the differing rates of mucin degradation is that extra enzymes are required to break down the colonic mucin. The original inoculum may have contained fewer bacteria producing such enzymes or they produced lower activities of enzymes than the enzymes required for gastric mucin degradation. Thus this becomes the rate-limiting step.

Hoskins and Boulding (1981) claimed that degradation of gastric mucins was a specialised function of less than 1% of the total faecal flora. No studies have reported specifically on the degradation of colonic mucin but the numbers of bacteria capable of degrading it may be orders of magnitude lower than those capable of gastric mucin degradation. Hoskins (1981) also suggested that fluctuations in the numbers of bacteria capable of degrading mucins may be important in intestinal disorders such as inflammatory bowel disease.
Enumerating mucin degrading bacteria using gastric mucin may miss an important subpopulation of bacteria capable of catalysing colonic mucin degradation.
5.5 **Difficulties in determining mucin degradation**

A number of "mucinase" assays have been published (Ross, 1959; Wiel-Korstanje *et al.*, 1975; Salyers *et al.*, 1977; Hoskins *et al.*, 1981) but comparisons between them are difficult because of the different methods and substrates that are used. Several aspects require comment in order to compare the results with those presented in this thesis.

Salyers *et al.*, (1977) did not consider the possible inducibility of bacterial enzymes when testing laboratory cultures that had not previously been grown on mucin. Wiel-Korstanje *et al.*, (1975) showed that degradation of mucin by even newly isolated bacteria was increased when the inoculum was grown on mucin. In addition laboratory cultures may lose their expression of mucin-degrading enzymes during subculture using monosaccarides as the energy source. A similar difficulty arises when monosaccarides are incorporated in the basal medium such as that used by Wiel-Korstanje *et al.*, (1975) and Hoskins *et al.*, (1981). The presence of glucose could suppress the production of mucin-degrading enzymes. An attempt was made to overcome these difficulties in this thesis by isolating and maintaining cultures only on the mucin substrates.

The definition and method of testing for mucin degradation is also a problem when comparing assays. Salyers *et al.*, (1977) tested for the production of fermentation products and this is also the basis of the assay used by Wiel-Korstanje *et al.*, (1977) which was investigated in this thesis (Appendix four).
Hoskins *et al.*, (1981) tested for the loss of mucin sugars and Ross (1959) tested for the loss of mucin precipitation by CPC. The ability of a bacterium to degrade mucin therefore can be a matter of degree as well as semantics.

It was shown in Section 4.1 that even the ability to cleave only one or two carbohydrate bonds of mucin may be significant, as in the utilisation of mucin by the type cultures of *Bacteroides fragilis*. This limited mucin-degrading ability may not have been detected by the fermentation method of Salyers *et al.*, (1977) thus accounting for why they failed to detect many bacteria capable of fermenting mucin. Therefore, in this thesis, the mucin degradation was quantified by determining both the percentage of each individual sugar that was cleaved as well as the molecular size of the degraded products.
5.6 Role of mucin degradation in the ecology of the gastrointestinal tract

Hungate (1966) noted that bacteria in the rumen could adapt into two major ecological niches for nutrition. They are either highly specialised and live on very few food sources or they are adaptable and live on a little of many different types of carbohydrate. This ecological reasoning might be aptly applied to the bacteria living in the mucus layer versus bacteria living in the lumen.

It was demonstrated (Section 4.1) that the type strains of *B. fragilis* were capable of growing to a limited yet significant extent on gastric and colonic mucin. *B. fragilis* is one of the more numerous organisms in the human colon and can produce many different glycosidases (Rudek et al., 1976). These bacteria in the lumen are exposed to a wide variety of dietary fibre components, epithelial cells and secretions. Therefore their enzymes must be capable of obtaining nutrition from as many of these food sources as possible without specialising into the degradation of any one. Because mucins have many different glycosidic linkages degradation of mucins by pure cultures is confined to only suitable terminal sugars. Complete degradation of mucins would need to be a co-operative process of many bacteria sequentially or competitively cleaving suitable exposed terminal residues on the carbohydrate chains.

Evidence for the fact that limited growth on glycoproteins could be a nutritional advantage in the colon was found by Hoskins and Boulding (1976). They showed that there was a 50,000 fold increase in the number of bacteria capable of cleaving
the B antigenic determinate (α-D-galactose) in type B secretors over A or H secretors. Similar evidence was also found by Miller and Hoskins (1981). They found that 10 of 17 cultures that showed moderate mucin degradation (51-57% of the hexose) could only hydrolyse either the A or H blood group determinants of gastric mucin. Therefore complete mucin degradation was by a co-operative process.

In contrast to bacteria in the lumen, the microbial flora of the mucosal surface is constantly surrounded by high concentrations of mucin (Davis, 1976). Bacteria that can almost completely degrade mucin have been isolated from the mucosal surface. (This thesis and Gustafsson, 1982). These bacteria must be specialised in degrading mucin and can produce many of the numerous enzymes required for complete mucin degradation. This specialisation in the pig colon bacteria is evident from the production of enzymes which cleave the outer chain fucose and neuramate residues from the oligosaccharide chains. The bacteria hydrolysed these sugars but preferentially utilised the underlying hexose and hexosamine residues.

It is not yet known how the balance between the degradation of mucus, by the mucosal bacteria, and mucus production is regulated. Further research is required to investigate if mucus degradation affects the role or roles of the mucus in the large intestine.
Physiological implications of mucin breakdown

As there are no accurate measurements of the quantities of mucin available to be degraded the relative importance of mucin as a food source to the bacteria in the colon is difficult to establish. It is likely to be a significant, even if minor, portion of the 60 grams of carbohydrate fermented in the human colon each day (Cummings, 1981). The anaerobic fermentation of the mucin produces volatile fatty acids (VFA's) as products. Most of the VFA's are absorbed through the mucosa and are metabolised (Cummings, 1981) thus recovering some of the energy expended in producing and secreting the mucins.

The interactions between mucins and bacteria may have more important implications to the host. Smith and Butler (1974) suggested that acidic mucin was important for colonic mucosal protection. Sakata et al., (1981) observed that there was less sulpho-mucin in the luminal mucin layer of the proximal colon than was in the epithelial mucin at the same site. They suggested that the difference was due to bacterial degradation of the luminal mucin. The investigations for this thesis have found evidence that would support the theory that Alcian blue reactive sulpho-mucins in the colon are less degradable than neutral or sialomucins. Degradation of the colonic mucin was slower than the gastric mucin. In vivo gastric mucin is not exposed to high numbers of bacteria because of the acid concentrations in the stomach. Resistance to bacterial degradation is therefore not required.
Other observations in the literature suggest that sulphate esters on the mucins might be an important part of the mucosal defence mechanism against bacteria. Fetal mucins only develop full sulphonyl-mucin staining towards parturition when the gut is first colonised by bacteria (Horowitz, 1977). In ulcerative colitis the mucin carbohydrate chains are shorter and less sulphated than normal (Clamp et al., 1981). Sulphated polysaccarides in the diet such as corcogeen, sulphated amylo-pectin and ligno-sulphate, can initiate a bacterial-dependent ulceration of the intestinal mucosa in rodents and monkeys (MacPherson et al., 1976). Bacterial colonisation of bladder surface mucin is prevented by the bacteria being bound to, and excreted with, the mucin. The binding can be inhibited by positively charged protamine and is duplicated by heparin sulphate suggesting that sulphate residues are important to the binding mechanism (Parsons et al., 1981).

These observations, and the findings of this thesis suggest that the role of sulphated mucins in mucosal protection warrants further investigation. Conclusive proof requires the availability of enzymes to specifically remove the sulphated residues and other sugars from the mucins and allow the appropriate control experiments to be carried out. Defining the mechanism of mucin resistance to degradation would also suggest further experiments to determine whether abnormalities in mucus composition and/or bacterial extracellular enzymic activity are important in pathological diseases of the gastrointestinal tract.
APPENDIX ONE

Bacterial contamination of Sigma gastric mucin

Introduction

Gram stains of uncentrifuged crude Sigma gastric mucin revealed the presence of bacteria. These organisms may have been present in harvested mucin or may have grown during extraction. The number of bacteria in the crude Sigma mucin was counted.

Method

Sigma gastric mucin (Lot 76C-0328) was dissolved at 10 mg per ml without prior purification. After dilution to 1 mg per ml the number of bacteria was determined in 0.01 ml by direct microscopic clump count (DMCC) using the procedure of the Virginia Polytechnic Anaerobe Laboratory Manual (4th Ed.).

Results and Discussion

A count of 10 fields under the phase contrast microscope (using the X 100 lens) revealed an average of 5 ± 5 (standard deviation) bacteria per field. Cellular debris was also present and prevented an accurate determination. Only obvious bacterial cells were counted but the number may have been higher. The gastric mucin therefore contains between $10^8$ and $10^9$ bacteria per gram (dry wt). If the mucin was extracted at a concentration of between 10 and 100 mg per ml then, in the order of $10^6$ bacteria per ml were present during processing.

The mucin from Sigma may therefore be partially degraded. Sigma gastric mucin has a low sialic acid content of approximately 12% (w/w). We have prepared gastric mucin from fresh mucosal
scrapings with a sialic acid content of 2\% (w/w) or higher. Allen and Starkey (1974) have found bacterial neuraminidase in pig gastric mucus. Thus it can not be discounted that sialic acid or other sugars may have been cleaved from the Sigma gastric mucin during processing.
APPENDIX TWO

Mucinase assay of Wiel-Korstanje and Winkler (1975)

Introduction

This "mucinase" assay was reported as a screening test to determine if an increased proportion of the faecal bacteria from ulcerative colitis patients could degrade mucin. No control experiments were published that tested the validity of the method. Roach and Tannock (1980) have also used the assay to test the mucin degrading ability of isolates from the mouse caecum.

We have carried out experiments to compare the results of the "mucinase" assay with the estimates of mucin degradation in this thesis, obtained using carbohydrate and Sepharose chromatographic analysis of the digested mucins.

Method of Wiel-Korstanje and Winkler (1975)

"Mucinase" was detected on Brain-Heart Infusion agar (Difco) to which 0.28% (w/v) of pig gastric mucin (Koch-Light Lab. Ltd., Colnbrook, Bucks.) had been added at 45°C before the plates were poured. After spot inoculation with bacteria and incubation for 48 h at 37°C, the plates were flooded with a solution of 1% calcium chloride. "Mucinase"-positive strains of bacteria produced clear zones around the inoculated spot. The authors suggested that mucin was precipitated by the calcium chloride in those areas where it had not been degraded by bacteria.

Bacterial cultures tested

Cultures 13 and 42 were isolated from the pig colon and have
been shown to extensively degrade Sigma pig gastric mucin (see Chapter 4). *Bacteroides fragilis* ATCC 25285 has been shown to grow on Sigma gastric mucin without significantly changing the molecular size (Section 4.1). Culture 111-6 was isolated in the laboratory of G. Tannock, Department of Microbiology, University of Otago, Dunedin and showed good positive results in the "mucinase" assay of Wiel-korstanje and Winkler (1975).

Two isolates of *E. coli* were supplied by H. Brooks, Department of Microbiology, Massey University. They were human urinary tract isolates. One strain (designated *E. coli* +ve) was capable of degrading ovomucin and the other was a control strain which did not degrade ovomucin (H. Brooks, personal communication).

**Method**

The procedure of Wiel-korstanje and Winkler was followed to test for mucinase activity, using 0.28% dialysed Sigma gastric mucin.

**Results and discussion**

A summary of the "mucinase" assay results is listed in Table 6.1. The *B. fragilis* culture produced a strong clear zone of no precipitation around the inoculated spot. This culture can grow on gastric mucin but cannot extensively degrade the carbohydrate chains. Culture 13 also produced an extensive clear zone on the agar. It can extensively degrade high-molecular weight gastric mucin to smaller sized fragments. Culture 42 can also degrade gastric mucin to small fragments. However, it did not produce a clear zone around the inoculated spot. Culture 111-6 produced a clear zone, while the *E. coli* strains did not.

The results show that the "mucinase" assay did not correctly reflect
the ability of the cultures to degrade high-molecular-weight mucin. Further investigations were done to determine the basis of the assay.

"Mucinase" penetration of dialysis membrane

Method

One centimetre squares of agar were aseptically cut from a plate of brain-heart infusion agar containing 0.28% mucin. The blocks were placed on top of an agar plate of similar composition with a double layer of boiled dialysis tubing between the surfaces. Cultures of B. fragilis ATCC 25285 and culture 111-6 were used to inoculate separate agar blocks. After 48 h of anaerobic incubation the dialysis tubing and agar blocks were removed. The bottom agar was then flooded with 1% CaCl₂.

Result and discussion

A clear zone without precipitation was visible where the inoculated blocks had rested on the dialysis tubing. No visible damage had been done to the dialysis membrane. The clearing factor was therefore of low molecular weight. This was suggestive of acidic fermentation products. A further experiment was carried out to test this.

Reversal of "mucinase" action by pH adjustment

Method

Brain-heart infusion broth (Difco) containing 0.28% Sigma gastric mucin was anaerobically sterilised and 3 ml aliquots dispensated. Tubes were inoculated in duplicate from fresh cultures and allowed to grow at 37°C for 2 days. The pH was then measured. One tube of each culture
was readjusted to pH 7.0 with 0.3 M NaOH. The "mucinase" assay was performed by adding 1 ml of 1% CaCl₂ to all tubes.

Results and discussion

The effect of adjusting the pH of the media is summarised in Table 6.2. When the pH was low no precipitation formed. However, if the medium was then neutralised this allowed the formation of a precipitate. When bacterial medium without mucin was titrated with 0.1 M acetic acid in the presence of CaCl₂, the precipitate dissolved with a sharp endpoint about pH 5.8 - 5.9.

Results of the mucinase assay must therefore be re-interpreted. The precipitation that is observed is probably that of calcium phosphate salts which are only soluble at acidic pH.

The clear zone around "mucinase" positive colonies in the assay would be caused by the diffusion of acidic fermentation products. Wiel-Korstanje and Winkler (1975) demonstrated that lactic acid was produced from mucin by four of their cultures that were "mucinase" positive. This confirms that the bacteria can degrade the mucins. The original "mucinase" test, however, was done using Difco brain-heart infusion agar. This medium contains 0.2% (w/v prepared media) of glucose. Acidic fermentation products could therefore be produced from this carbohydrate as well as from the mucin, giving false positive results.

Wiel-Korstanje and Winkler (1975) also showed that lactic acid production from mucin was inducible. The glucose in the brain-heart infusion test medium may repress the production of "mucinase" enzymes in some bacteria.
Conclusions

The "mucinase" assay of Wiel-Korstanje and Winkler (1975) does not necessarily detect the splitting of mucin molecules to smaller units as stated by the authors. It can detect the production of acidic fermentation products. The fermentation products, however, may be partly or wholly derived from glucose in the medium.
<table>
<thead>
<tr>
<th>Culture</th>
<th>Effect of CaCl&lt;sub&gt;2&lt;/sub&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>13</td>
<td>Large clear zone</td>
</tr>
<tr>
<td>42</td>
<td>No effect</td>
</tr>
<tr>
<td>111-6</td>
<td>Clear zone</td>
</tr>
<tr>
<td><em>E. coli</em> + ve</td>
<td>No effect</td>
</tr>
<tr>
<td><em>E. coli</em> - ve</td>
<td>No effect</td>
</tr>
<tr>
<td><em>B. fragilis</em> ATCC 25285</td>
<td>Clear zone</td>
</tr>
</tbody>
</table>
Table 6.2  Effect of pH on the mucinase assay of Wiel-Korstanje and Winkler (1975)

<table>
<thead>
<tr>
<th>Culture</th>
<th>pH after incubation</th>
<th>Effect of CaCl₂</th>
<th>pH after neutralisation</th>
<th>Effect of CaCl₂</th>
</tr>
</thead>
<tbody>
<tr>
<td>Not inoculated</td>
<td>7.0</td>
<td>Sediment</td>
<td>7.0</td>
<td>Sediment</td>
</tr>
<tr>
<td>B. fragilis</td>
<td>5.9</td>
<td>No effect</td>
<td>7.0</td>
<td>Sediment</td>
</tr>
<tr>
<td>Culture 42</td>
<td>6.4</td>
<td>Partial sediment</td>
<td>7.0</td>
<td>Sediment</td>
</tr>
<tr>
<td>Culture 13</td>
<td>5.7</td>
<td>No effect</td>
<td>7.0</td>
<td>Sediment</td>
</tr>
<tr>
<td>E. coli</td>
<td>6.0</td>
<td>Sediment</td>
<td>7.0</td>
<td>Sediment</td>
</tr>
</tbody>
</table>
APPENDIX THREE

Labelling of mucins with Remazol brilliant blue

Remazol brilliant blue is a Procion type dye which will react with hydroxyl groups by an esterification reaction. It can attach to the C2, C3 and C6 groups of cellulose and is used as a cotton textile dye and as a histological protein stain. By labelling the C2 position of glucose in starch or glycogen it can also be used to monitor amylase activity as the position of the label does not interfere with enzymic activity (Rinderknecht et al., 1967).

In histology, Remazol brilliant blue will stain protein but not the PAS negative hyaluronic acid or chondroitin sulphate. This is thought to be due to the presence of sulphate-substituted hydroxyl groups (Marshall, 1966). Because the mucins are PAS reactive an attempt was made to label mucins with Remazol brilliant blue and determine if specific glycosidases could then remove the label.

Method

Remazol brilliant blue was obtained from Serva (Feinbiochemicia, Heidelberg, Germany) and used according to the procedure of Stamm (1963). Dialysed Sigma gastric mucin and pig gastric mucin extracted by tryptic digestion in this laboratory were dissolved at 10 mg per ml in distilled water. The mucins were mixed with equal volumes of Remazol brilliant blue in water (10 g/l) and heated to 50°C in a water bath. While continuously stirring, 0.05 gm of sodium sulphate was slowly added, and the mixture stirred at 50°C for 45 min. This solution was then treated by adding enough trisodium phosphate to give a final concentration of 5 g/l and the solution stirred at 50°C for another 75 min. The whole sample was then dialysed against 0.2 M NaCl for 5 days at 4°C. After this time dialysis tubing that
originally contained only a 1% dye solution had gone completely colorless while the tubing containing labelled mucins was still blue with optical densities at 620 nm of 6.0 for the Sigma gastric mucin and 4.7 for the gastric mucin prepared from mucosal scrapings.

Results and discussion

A 1 ml sample of each of the labelled, dialysed mucin solutions was run on a Sepharose 4B column. The samples were eluted with 0.1 M NaCl containing 0.02 M sodium phosphate buffer, pH 7.0. Greater than 80% of the blue colour eluted in the low-molecular-weight fractions of both preparations with optical densities at 620 nm of less than 0.10 O.D. The normal PAS colorimetric assay (Mantle and Allen, 1978) was also performed directly on the 2 ml fractions. Peak optical densities of greater than 0.8 O.D. at 555 nm confirmed the presence of high molecular weight, periodate-oxidisable mucins. Glycogen samples were intensely labelled by the dye using the same procedure.

The results obtained are therefore conflicting and do not agree with the hypothesis of Marshall (1966) that the failure to stain mucosubstances is due to the presence of sulphate-substituted hydroxyl groups. Periodate oxidisable hydroxyl groups were present in the mucin samples but did not react with the dye. The procedure was tried only once on the mucins but the labelling of glycogen, as well as the contaminating protein in the mucin, act as positive controls for the method.

The hydroxyl groups of the mucin may not be accessible to the dye because of the charged and hydrated nature of the molecule. Alternatively the neuraminic acid, fucose and galactose residues on
the mucins, which have free hydroxyl groups, may not form stable ester linkages with the dye.
APPENDIX FOUR

Development of the colonic mucin extraction

Introduction

The immediate goal of this thesis was to extract mucin glycoprotein from the pig colon for use as a model substrate in studies of mucus degradation by bacteria. The ideal extraction and purification procedure would be one that recovered gram quantities of mucin glycoprotein in high yield and without degradation or conformational changes. The extensive studies by Allen's group (Allen and Snary, 1972; Snary and Allen, 1972; Allen and Starkey, 1974; Marshal and Allen, 1977) at the University of Newcastle-upon-Tyne, served as the model and starting point.

The method used to extract the mucin was developed and improved over a period of three years. The most efficient procedure devised and adopted for all later experiments is given in the methods section. This discussion is limited to the observations and findings on which that method is based.

Observations on the site of colon mucus collection

The initial trial of extraction was performed using mucosal scrapings from the pig caecum which has a large flat surface area to collect from. All subsequent collections were from the colon, and all bacterial experiments were performed on mucus collected from the junction of the centripetal and centrifugal coils.
This area could be easily and rapidly identified. Approximately 60 cm was quickly excised without removing the whole viscera from the moving offal table. The uniformity of collection site is important in view of the observations of Sakata et al. (1981) and Spicer (1965) that mucin composition changes along the gastrointestinal tract.

The age of the pigs was highly variable and ranged from "porkers" (young) to "baconers" (mature) and occasional "choppers" (ex-breeding animals). Considerable variations in diet were also noticeable from the gut contents as were variations in the thickness of the mucosal wall, the viscosity of the mucus collected and the general appearance of the mucosal surface. Thick mucosa and viscous mucus tended to be associated with colons containing hard faecal material. Diverticular-like pocketing in some colons made mucus collection difficult. Between 0.5 and 2.0 ml of mucosal scrapings could be collected from each colon section and all samples were randomly pooled for later processing.

Sialic acid as a marker to quantitate mucin

As mucus is composed of many different components the accurate assay of mucin in the mucosal scrapings was difficult. Without radioactively-labelled sugars, as used by Snary et al. (1972), specific sugar analysis was the only feasible option. The anthrone reaction was found to be unusable for the assay of crude preparations. Strong interference was observed, presumably from the high content of contaminating protein (Spiro, 1966).
The assay of neuraminic acid by the method of Warren (1959) proved to be a specific and easily applied determination. Greater than 90% of the neuraminate was bound to high molecular weight mucin that was excluded on Sepharose 6B. If sialic acids were present that were attached to low molecular weight cell-surface glycoproteins, then they constituted a very minor portion of the total content.

An advantage of using neuraminate as a marker was its sensitivity to bacterial hydrolysis. Because of its terminal position, removal of neuraminate is a prerequisite to mucin degradation (Forstner, 1978). The isolation of mucin with a high content of neuraminate was therefore indicative of an undegraded glycoprotein. If bound neuraminate was lost during mucin extraction then enzymic or bacterial degradation had occurred.

**Aqueous extraction only**

Many attempts were made to solubilise the colonic mucin glycoprotein into aqueous solution with only partial success. The mucus was diluted into 2 volumes of 0.1 or 0.2 M NaCl and homogenised either by blending for up to 2 min in a Waring-type blender, or by making several passes in a motor-driven Thomas Teflon-pestle tissue grinder. The soluble glycoprotein was then separated from the insoluble gel by centrifugation at 10,000 x g for 10 min. Between 10% and 15% of the total neuraminic acid could be solubilised in this manner. If the precipitated insoluble gel was re-homogenised into another
2 volumes of 0.1 M sodium chloride, a further 5-6% of the neuramine remained in solution after centrifugation. In 6 determinations on separate collections of colonic mucosal scrapings no more than 21% of the glycoprotein was solubilised by aqueous extraction alone.

Other investigators have noted low recoveries of total mucins from aqueous solution. Snary and Allen (1972) could only solubilise 20-27% of gastric mucin by similar methods, but their later papers (Robson et al. 1975; Mantle and Allen 1981) claim total solubilisation of gastric mucus gels into aqueous solution by homogenisation. The difference might be accounted for by the use of low mucin concentrations. High dilution may avoid mucin-chain interaction and subsequent precipitation on centrifugation. La Mont and Ventola (1980) extracted rat colonic mucus by aqueous, non-degradative methods. They noted that a large percentage of the mucin was lost in the initial centrifugation.

Marshall and Allen (1978) reported the isolation of pig colonic mucin by homogenisation of mucosal scrapings. They found that the water-soluble mucus constituted 75% of the total dry weight of the mucosal scrapings and, of that, only 5% was glycoprotein. They filtered the mucosal scrapings through a muslin cloth to remove cell debris. This procedure was tried in this laboratory but most of the insoluble mucus gel appeared to be retained by the cloth. The bulk of the glycoprotein was subsequently found in the insoluble mucus gel. Therefore only centrifugation was used to separate the soluble and insoluble mucus fractions.
Trials of other methods to solubilise mucus

Experiments were conducted to try and maximise recovery of the colonic mucin from the insoluble gel. Mucosal scrapings were homogenised in 0.1 M NaCl using a Potter teflon-pestle homogeniser. The solution was centrifuged at 10,000 x g for 10 min and the precipitate rehomogenised in 6 M urea. A sol/gel phase separation was evident after further centrifugation with the top of the precipitate being clear and jelly-like. Less than 10% of the neuraminic acid was recovered in the supernatant fraction indicating that the bulk of the mucin was still insoluble.

Mucosal scrapings were also collected into 5% (w/v) EDTA and 20% (w/v) CaCl₂ solutions. Neither agent was effective in preventing the formation of a large gel phase which precipitated upon centrifugation. The use of degradative methods to solubilise the mucus gel was therefore accepted as inevitable.

A series of trials were carried out to determine the best combination of agents that other investigators (Snary and Allen, 1972) had found effective in solubilising mucus gels. Mucosal scrapings from the colon were homogenised at 4°C with 2% (w/v) NaN₃ to give a final concentration of 0.02% azide. Tubes of this mucus that were incubated for 12 h at room temperature showed the release of free neuraminic acid. Tubes left at 4°C did not. The presence of significant neuraminidase contamination meant that it was likely that there were other bacterial glycosidases in the mucosal scrapings. To eliminate most of the soluble enzymes and proteins all further experiments were done
on washed, insoluble mucus. The scrapings were twice homogenised in 0.1 M NaCl, 0.01 M phosphate buffer, pH 7.0 and centrifuged. Soluble proteins were discarded with the loss of approximately 20% of the mucin glycoprotein.

Residual neuraminidase activity was still present in the insoluble mucus but did not interfere with small scale trials of trypsin and mercaptoethanol extraction. The washed mucus preparation (2 ml) was suspended in 1 volume of 0.1 M NaCl, 0.02% NaN₃, 0.01 M phosphate buffer pH 7.5. Trypsin treatment (0.7 mg/ml) at 30°C for 15 h resulted in approximately half the neuraminic acid being left in solution after centrifugation at 10,000 x g for 3 min. For comparison two milliters of the same mucus solution was treated with 0.1 M β-mercaptoethanol at 30°C for 15 h. This left 95-100% of the neuraminic acid in the supernatant.

Mercaptoethanol extraction

Washed, insoluble colonic mucus gel was processed by treatment in 0.5 M mercaptoethanol, 0.02% NaN₃, 0.1 M sodium phosphate buffer, pH 7.5 at 0°C. After overnight incubation the gel was visibly dissolved and 95-100% of the neuraminic acid was found to be in solution after centrifugation at 10,000 x g for 10 min. The supernatant fraction was centrifuged again at 26,000 x g for 150 min. A small pellet was formed by this high-speed run but it contained less than 5% of the neuraminic acid in the total fraction. Optical densities at 549 nm and 532 nm in the neuraminic acid assay (Warren 1959) indicated that most of the DNA had been centrifuged out of solution.
The remaining supernatant was dialysed against distilled water and freeze dried. However less than half the neuraminic acid containing material could then be redissolved into 0.1 M NaCl. The disulphide bonds may have reformed thus polymerising the mucin as an insoluble aggregate.

Scawen and Allan (1975) had shown that the disulphide bonds of pig gastric mucus could be removed by proteolytic digestion without loss of the sugar residues. The glycosylated regions of the subunit peptides are protected from degradation. This procedure was therefore adopted to remove the disulphide bonds rather than sulphydryl blocking reagents. Contaminating enzymes and other proteins would also be fragmented and could then be dialysed out.

Prevention of bacterial growth

Inoue et al (1966) and Murty et al (1978) boiled the insoluble colonic mucus before extraction. To avoid such harsh treatment, the first studies used tyndalisation to kill bacteria and inactivate enzymes. Even with the presence of toluene in the solution this procedure was found to be unreliable if only done once every 24h.

Sodium azide (0.02%) was tried as a sterilising agent and proved completely effective in preventing bacterial growth, provided the solution was kept aerobic. Therefore the tyndalisation procedure was deleted but residual glycosidase activity was then found in the final preparation despite trypsin digestion.
A trial showed that more than 90% of the neuraminidase activity in fresh mucosal scrapings could be destroyed by 15 min heating at 60°C. The insoluble mucus was therefore heat-treated at 80°C for 30 minutes. Samples, before and after the trypsin digestion step, were extracted with methanol to separate the free and bound sugars. No free hexosamine and less than 10 μg/ml of free hexose or fucose were found in these samples that were estimated to contain approximately 6 mg/ml of mucin. The heat treatment is therefore effective in preventing glycosidase action during the extraction procedure.
Use of gamma-irradiation for sterilisation

An attempt was made to sterilise the fresh mucus by exposure to irradiation using a 0.4 Mrad per hour $^{60}$Co source. In an initial trial fresh mucus gel was degraded by the contaminating bacteria before sterilisation was achieved. Freeze-dried mucus gel was therefore used to determine the necessary radiation exposure for complete sterilisation.

A sample was irradiated in a sealed glass test-tube and a loopful periodically inoculated onto chopped-meat carbohydrate agar (V.P.I. Anaerobe Laboratory Manual, 4th Edit. p.144). The agar plate was incubated anaerobically for two days and then examined for growth. After 1 Mrad exposure there were still many viable bacteria. At 2 Mrad exposure only four single colonies were observed. Therefore at least 4 Mrad radiation would be needed to ensure complete sterilisation. At this level of exposure there was visible disintegration and colouring of the mucus. Radiation was therefore not used to sterilise mucus.

The $^{60}$Co source was useful to treat non-autoclavable kid-leather gloves. These were used inside the anaerobic chamber to handle sterile materials. They were given at least 4 Mrad radiation exposure inside a resealable plastic bag which was not opened until the gloves were required for use.
APPENDIX FIVE

Calculation of sedimentation coefficients by method of Martin and Ames (1961)

The mucin preparations were sedimented on a sucrose-density gradient as detailed in Section 3.1. The radial displacement of each fraction, the centrifugation speed and time were then used to calculate a sedimentation coefficient by the method of Martin and Ames (1961). A partial specific volume of 0.64 mg per ml was assumed for all the fractions. This was the value estimated by Marshall and Allen (1978) for pig colonic mucin. The calculations were done using a BASIC language computer program (Dr R. Geddes, Department of Biochemistry, University of Auckland, N.Z.).

The most concentrated fraction of colonic mucin (Table 6.3) had an $S^{20,w}$ value of 34 S. This preparation had been isolated with mercaptoethanol treatment followed by tryptic digestion and therefore should be a subunit of the original native mucin. (Marshall and Allen 1978.) The most concentrated fraction of the gastric mucin preparation that remained in solution had an $S^{20,w}$ value of 18 S. This is also a mercaptoethanol treated and trypsin digested preparation. Sigma gastric mucin showed a continuous increase in optical density to the bottom of the tube. The peak fraction was therefore not determined but it would have a calculated sedimentation coefficient greater than 70 $S^{20,w}$. This size, however, is inconsistent with its method of isolation as it is extracted from a Pronase digest of gastric mucosal scrapings. (Personal communication from Sigma Chemical Co. Missouri, U.S.A. to Dr S.P.Lee, Department of Medicine, University
either the preparation was not completely digested to subunits during isolation or subsequent aggregation occurred.

These calculated sedimentation coefficients however are only in approximate agreement with those of Marshall and Allen (1978) and Starkey et al. (1974).

Marshall and Allen (1978) found an $S_{25,w}^0$ value of 13.5 S for the Pronase digested, mercaptoethanol-treated pig colonic mucin. Starkey et al. (1974) found a sedimentation coefficient of 15 $S_{25,w}^0$ for the smallest subunit of gastric mucin that was produced by either mercaptoethanol treatment or Pronase digestion. These values are smaller than those calculated for the colonic and gastric mucin preparations using the sucrose-density centrifugation data.

Two possibilities exist to explain the discrepancies. The first is that trypsin, because of its greater site specificity, does not cleave the molecule at the same regions as the Pronase that was used by Marshall and Allen (1978). This is unlikely as the gastric and colonic mucin preparations eluted in the included volume of the Sepharose 4B gels (Figs. 3.9 & 3.6). By direct analogy of the sedimentation coefficient with those obtained by Marshall and Allen (1978) the colonic mucin preparation would have a molecular weight of $6 \times 10^6$ and should thus be excluded by Sepharose 4B gel.

The second possibility is that the sedimentation coefficients determined using the dilute PAS labelled mucins are higher than
those obtained during conventional sedimentation - velocity analysis. Jabbal et al (1975) found close agreement between results from conventional boundary centrifugation and band ultra centrifugation analysis using dilute PAS-stained mucins. However, the band ultra centrifugation was done in 0.18 M KCl as opposed to the essentially zero ionic strength of the sucrose gradients used in this investigation. Below 10 mM NaCl the intrinsic viscosity of isolated mucin glycoproteins increases about tenfold (Allen, 1978). This could affect the calculation of sedimentation coefficients by the method of Martin and Ames (1961) which assumes that no intermolecular interactions are taking place. Therefore this is the most probable cause of the apparently high sedimentation coefficients calculated from the sucrose-density centrifugation data.

In conclusion the sucrose density centrifugation was useful to show the unimodal, but heterogenous nature of the colonic mucin preparation. By using lower concentrations of mucin and higher ionic strength in the gradient the method could also be used to rapidly obtain sedimentation coefficients on less than a milligram of mucin without the use of an analytical ultracentrifuge.
<table>
<thead>
<tr>
<th>Fraction No.</th>
<th>Refractive Index</th>
<th>Sedimentation Coefficient (S°&lt;sub&gt;20, w&lt;/sub&gt;)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.4187</td>
<td>68</td>
</tr>
<tr>
<td>2</td>
<td>1.4137</td>
<td>63</td>
</tr>
<tr>
<td>3</td>
<td>1.4074</td>
<td>48</td>
</tr>
<tr>
<td>4</td>
<td>1.4010</td>
<td>40</td>
</tr>
<tr>
<td>5</td>
<td>1.3962</td>
<td>34</td>
</tr>
<tr>
<td>6</td>
<td>1.3915</td>
<td>29</td>
</tr>
<tr>
<td>7</td>
<td>1.3870</td>
<td>25</td>
</tr>
<tr>
<td>8</td>
<td>1.3837</td>
<td>21</td>
</tr>
<tr>
<td>9</td>
<td>1.3802</td>
<td>18</td>
</tr>
<tr>
<td>10</td>
<td>1.3773</td>
<td>15</td>
</tr>
<tr>
<td>11</td>
<td>1.3742</td>
<td>13</td>
</tr>
<tr>
<td>12</td>
<td>1.3712</td>
<td>10</td>
</tr>
<tr>
<td>13</td>
<td>1.3682</td>
<td>8</td>
</tr>
<tr>
<td>14</td>
<td>1.3645</td>
<td>6</td>
</tr>
<tr>
<td>15</td>
<td>1.3610</td>
<td>4</td>
</tr>
<tr>
<td>16</td>
<td>1.3589</td>
<td>2</td>
</tr>
</tbody>
</table>

* Calculated by the method of Martin and Ames (1961). The density of the solution, centrifugation speed, time and the radial position of the fraction are used to calculate an approximate sedimentation coefficient assuming a partial specific volume of 0.64 gm per ml for all mucins.

* Fractions were collected from the bottom of the tube.
REFERENCES


Hoogkamp-Korstanje, J.A.A., Lindner, J.C., Marcelis, J.H.,
den Daas-Slagt, H. and de Vos N.M. (1979) Antonie van
Leeuwenhoek, 45, 35-40.

and Pigman, W., Eds.) pp 189-209, Academic Press, New York,
San Francisco, London.

and Pigman, W., Eds.) pp 231-253, Academic Press, New York,
San Francisco, London.


New York and London.

Hungate, R.E. (1969) in Methods in Microbiology Vol.3B (Norris, J.R.
and London.


117, 257-265.


Scott, J.E. and Dorling J. (1965) Histochemie, 5, 221-223.


