THE PARTIAL CHARACTERIZATION OF THE EPITHELIAL GLYCOPROTEIN FROM NORMAL AND DISEASED COLONS

by

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We accept this thesis as conforming to
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ABSTRACT

This study reports the isolation, fractionation and chemical characterization of apparently homogenous and undegraded epithelial glycoproteins from normal human colons and from patients with ulcerative colitis, Crohn's disease and ischaemic colitis of the colon. Colonic epithelial cells were removed from each specimen by shaking with EDTA and the glycoproteins were extracted from the sonated cells with 1M NaCl. Combined agarose gel and DEAE cellulose chromatography of the pooled crude extract from each normal and diseased sample yielded two apparently homogeneous fractions A and B. Agarose gel electrophoresis revealed that the mobility of the second DEAE fraction (B) was about the same as that of heparin and twice as fast as that of fraction A. Application of gas-liquid chromatography, UV-spectroscopy, agarose gel electrophoresis at pH 3.0, colorimetric hexose analysis, phosphorus and Indole assays suggested that fraction B was DNA.

Fraction A glycoproteins appeared to be homogeneous by DEAE chromatography, agarose gel and cellulose acetate electrophoreses at pH 8.8. Chemical analyses of these glycoproteins revealed the presence of fucose, galactose, hexosamine, sialic acid, protein and little or no phosphorus. The molar ratio of the carbohydrate components of each sample was significantly

different from that of the normal, and it varied among the different diseases. In each of the glycoproteins studied, 4 different types of sialic acids were found in different quantities and they were: (1) unsubstituted or C9 substituted sialic acids, (ii) sialic acids substituted at C4 and or at C9, (iii) sialic acids substituted at C7 and or C8, (iv) sialic acids substituted at C4 and at C7 and or C8. Gas-liquid chromatography revealed that these substituents were alkaline-labile O-acetyl groups. The significance of the above differences were discussed.

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INTRODUCTION

The research described in this thesis concerned the isolation and characterization of epithelial glycoproteins from normal and diseased human colons. Chemical and histochemical evidence suggested that glycoproteins play a role in intestinal diseases[1,2] and it was felt that such a study could contribute to a better understanding of the etiology and pathogenesis of colonic diseases particularly ulcerative colitis and Crohn's disease.

The chemistry of glycoproteins has been extensively reviewed by Gottschalk[3]. Glycoproteins are best defined as "conjugated proteins containing as prosthetic group(s) one or more heterosaccharide (s), the latter are usually branched, with a relatively low number of sugar residues, lacking a serially repeating unit and bound covalently to the polypeptide chain[4]. A major portion of the carbohydrate in animal tissues is found in conjugated form with proteins. Glycoproteins are a diverse group of compounds since the covalent association of carbohydrate with protein occurs in a wide range of protein types. While they have no unique amino acid composition, they do contain a characteristic group of sugars that include D-galactose, D-mannose, D-glucose, L-fucose, D-xylose, N-acetyl-D-glucosamine, N-acetyl-D-galactosamine and various derivatives of neuraminic acid (the sialic acids). The carbohydrate content of these glycoproteins may vary from

less than one percent to more than eight percent of the weight of the molecule, and as many as seven sugar types may be present in any given protein."

There is considerable confusion in the terminology used to describe macromolecules whose composition is in accord with this definition. Terms that have been employed include: mucin, mucoprotein, mucopolysaccharide, mucosubstance, sulphomucin, fucomucin, sialomucin, carboxymucin, acid mucopolysaccharide, glycosaminoglycan and glycoprotein. In this thesis, only two terms will be used to describe these types of macromolecules - (a) glycoprotein and (b) glycosaminoglycan. Any macromolecules which contains one or more of the following carbohydrate polymers: hyaluronic acid, heparin sulphate, keratan sulphate, dermatan sulphate and chrondroitin 4 and 6 sulphate will be termed glycosaminoglycans; all other macromolecules will be designated glycoproteins. According to this classification glycosaminoglycans (with the exception of keratan sulphate) contain uronic acid as a major carbohydrate constituent while glycoproteins normally contain sialic acid. Glycosaminoglycans are mainly found in connective tissues while the glycoproteins discussed here are assumed to be of epithelial origin.

Ulcerative colitis is a disease of unknown etiology. It is a diffuse, non-specific inflammatory condition of the

mucous membrane of the large intestine. The inflammation is of an exudative and vascular type and generally unproductive of granulation tissue or fibrosis. Although this disease can occur for the first time at any age, the peak age of incidence for either sex on first attendance at hospital is in the third decade. It has been estimated that the risk of cancer in patients with ulcerative colitis is between five and ten times greater than the risk in the general population [5,6]. Changes in the epithelial glycoproteins have been demonstrated both histochemically and chemically in this disease [7-10], but whether these changes are a cause or a consequence of the disease is unknown.

Chemistry of the Intestinal Glycoproteins

Viscous secretions of the epithelial cells are found throughout the gastrointestinal tract[11]. These secretions exist as highly hydrated gels the apparent functions of which are to lubricate the mucous membrane, to protect it from mechanical and chemical damage and from attack by enzymes and bacteria[12]. Chemically they are glycoproteins. It is speculated that the carbohydrate moiety forms a physical barrier which prevents proteolysis of the protein core (3). Thus, the integrity of the carbohydrate moiety may be essential for the survival of the glycoprotein, which in turn imparts protection to the mucosal epithelial cells.

There have been surprisingly few studies of intestinal glycoproteins. The procedures employed in the isolation and fractionation of these glycoproteins have been reviewed by Gottschalk[3], Marshal[13], Horowitz [14], and Spiro[1,2]. Three types of starting material have been employed for the extraction of these glycoproteins: (a) mucosal scrapings[11, 15-22], (b) minced colon[23,24] and (c) rectal irrigates[25,26]. The use of such starting materials make it unlikely that compounds of purely epithelial origin were isolated since scrapings and minced organs may contain connective tissue contaminants such as the glycosaminoglycans found in some studies[15,16,20-22], while irrigates could be contaminated with the products of faecal stream bacteria and enzymatic degradation of glycoproteins. In many cases, native glycoproteins were not obtained because proteolysis was used in the isolation procedure. Thus, it is difficult if not impossible to relate any of the structural data obtained to a specific glycoprotein.

Chemical analyses of the intestinal glycoproteins of several species[9,11,15-17,23] revealed the presence of protein, sialic acid, fucose, hexosamine and galactose. Only in the case of sheep colonic mucin [11] was an attempt made to perform a detailed structural analysis. Analytical data of limited scope was reported in the other studies. Thus, very little is known about the chemical and macromolecular structure of the intestinal glycoproteins of man and animals.

Animal Glycoprotein Studies

Werner[15] studied the composition of washed mucosal scrapings from gastro-intestinal tissues of the pig. Glycoproteins were shown to be a major component of the various mucins. On the basis of the analytical data, Werner postulated the existence of two different intestinal glycoproteins, one rich in fucose, and the other rich in sialic acid.

Inoue and Yosizawa[16] examined the glycopeptides obtained by the digestion of pig colonic mucosal scrapings with pronase. All the fractions obtained were acidic and contained O-sulphate ester. They could be separated into two groups: glycopeptides and glycosaminoglycans. The glycopeptides (molecular weight 2-3 X 10^5) contained galactose, fucose, glucosamine, galactosamine, sialic acid and sulphate. The glycosaminoglycan fractions, present in much smaller amounts than the glycopeptides, contained heparin sulphate, heparin and chondroitin sulphate. It was assumed that the glycosaminoglycans represented connective tissue contaminants. Nemoto and Yosizawa [17] obtained similar mixed fractions from pronase digests of mucosal scrapings of rabbit colon and small intestine. Glycosaminoglycans have also been found in mucosal scrapings of dog colon[18].

Kent et al[11] have carried out detailed studies on the water soluble glycoproteins obtained from scrapings of sheep colonic mucosa. Four macromolecular fractions were found, with sedimentation coefficients of 9.58, 6.1, 3.25 and 2.15 respectively. The major fraction (9.58S) was a sialic acid-containing glycoprotein with a molecular weight of about 2×10^6. Incubation of this fraction with papain resulted in a rapid reduction in viscosity without any loss of dialysable carbohydrate, and the production of a partially degraded glycoprotein of molecular weight of 1×10^5. The second fraction (6.1S) was substantially free of sialic acid, and could be degraded by papain to a 1.7S fraction (molecular weight 2×10^4). The remaining two fractions (3.2S and 2.15S) contained mainly nucleoproteins.In subsequent studies[27,28] an isotope labelled 9.58S glycoprotein was isolated from scrapings of sheep colonic mucosal tissue which had been incubated in Krebs-Ringer buffer for 4 hours in the presence of oxygen and such metabolites as D-(1-^{14}C) and D-(2-^{14}C) glucose, L-(U-^{14}C) threonine and $^{35}SO_4^{2-}$. Neither the 6.1S glycoprotein nor the two nucleoprotein fractions were labelled under these conditions. Chemical analysis of the 9.58S glycoprotein showed that it contained 25% protein, 72% carbohydrate and 4.6% ester sulphate. The sialic acid residues of the fraction, representing 13.7% of the glycoprotein, were made up of 63% N-glycollylneuraminic acid and 37% N-acetylneuraminic acid. The ease with which these residues could be hydrolyzed by dilute acids or neuraminidase implied that they occupied terminal positions in a "branched

oligosaccharide structure"[29]. Sequential "Smith degradation" of the ^{35}S-labelled 9.58S glycoprotein indicated that this glycoprotein had a peptide "backbone" to which an array of oligosaccharides (sialic acid-galactose-amino sugar or fucose galactose-amino sugar) were attached, probably through β-aspartamido linkages. Some of the amino sugar residues adjacent to the peptide chain were sulphated.

A glycoprotein containing sulphate and sialic acid was extracted from mucosal scrapings of rat small intestine with isotonic saline [19]. Chromatography of this salt-soluble glycoprotein on a DEAE cellulose column gave three fractions. The major fraction was carbohydrate-rich and contained sulphate. On ultracentrifugation and disc gel electrophoresis this fraction was resolved into two components, which could be partially separated by Sepharose-4B chromatography. Both subfractions had the same chemical composition as the parent mixture. Galactosamine, glucosamine, fucose, galactose and sialic acid were present in all fractions. The amount of sulphate detected was 2.4%, 40% of which was acid labile. Both sialic acid and fucose were rapidly released by acid hydrolysis suggesting that they occupied terminal positions. Threonine, serine and proline made up 69% of the total amino acid residues. The results obtained from alkaline borohydride treatment of the glycoprotein strongly suggested that galactosamine residues were linked to the peptide through O-glycosidic linkages involving serine and threonine residues.

Forstner et al[20] isolated a high-molecular-weight glycoprotein (HMG) from mucosal scrapings of rat small intestine. Antibodies against HMG were prepared in rabbits and the fluorescein-labelled antisera so obtained was found to stain the supranuclear mucous vesicles of goblet cells in the body of the stomach and mucus-producing cells in the sublingual gland. It was concluded that HMG was a major component of rat small intestine goblet-cell mucus. In a later study[21], HMG was found to be a negatively charged macromolecule with a molecular weight of about 2 X 10^6 and contained 12% protein, 23% hexose, 22.4% hexosamine, 10% sialic acid, 6.6% fucose and less than 1% sulphate. One major and two minor components were identified by acrylamide disc gel electrophoresis and by analytical ultracentrifugation.

Human Glycoprotein Studies

Roelf et al[25], have examined human rectal irrigates. Gas chromatographic analysis of hydrolysates of the irrigates revealed the presence of fucose, mannosamine, galactosamine, glucosamine, N-acetyl-neuraminic acid and N-acetyl-galactosamine. The presence of mannosamine was thought to represent the epimerization of glucosamine as a consequence of heating with pyridine.

Two rectal mucous plugs, obtained from children with cystic fibrosis of the pancreas and relatively uncontaminated by materials such as faeces and blood, were examined by Johansen [26]. The samples were insoluble in a wide variety of solvents including water, 3,6, and 9 M urea, formamide, saturated calcium chloride, ethanol (9:1 v/v), 0.1 M cysteine, salt solutions and mineral acids. They dissolved slowly in 0.1 M sodium hydroxide. Both samples contained galactose, fucose, sialic acid and N-acetylglucosamine. One sample contained traces of mannose. Uronic acid was not found in either sample. Sialic acid appeared to occupy a terminal position, as it could be removed from the oligosaccharide chain by vibrio cholera neuraminidase. Both glycoproteins were found to be resistant to trypsin, chrymotrypsin, pepsin and lysozyme, but could be dispersed with papain.

Sky-Peck et al[23] have isolated glycopeptides and glycosaminoglycans from proteolytic digests of whole human colon. Human colons were minced and the crude homogenate was incubated with papain at 60^{o}C for 24 hours. Treatment of the incubate with 1% cetylpyridinium chloride, (CPC), to precipitate the glycosaminoglycans present, yielded a CPC complex and a supernatant. Treatment of the material obtained from the CPC complex with testicular hyaluronidase followed by fractionation of the digest by a combination of Biogel P30 and DEAE

cellulose chromatography yielded a major hexose-rich fraction free of uronic acid. This material appeared to be homogenous by ultracentrifugation and free boundary electrophoresis at pH 8.4 and at pH 4.6. Chemical analysis revealed the presence of hexose, sialic acid, hexosamine and fucose. The supernatant from the CPC precipitation contained hexose. On fractionation on DEAE cellulose chromatography it yielded three distinct fractions. Galactose, mannose, hexosamine, fucose and sialic acid were present in each of these fractions. Hexuronic acid could not be detected. In two of the fractions hexosamine was present only as glucosamine while the third contained only galactosamine.

Korhonen and Makela[22] removed human colonic mucosa at the level of the lamina muscularis mucosae. The tissue obtained was dried at 60°C for 24 hours, homogenized, and then heated for 20 minutes in a boiling water bath. Pronase digestion of the heated homogenate was carried out at 60° for 48 hours and the glycopeptides obtained were extracted with trichloroacetic acid. Electrophoresis of the extract revealed the presence of four components, three of which contained sulphate. All these glycopeptides contained protein, hexose, fucose, hexosamine, sialic acid and uronic acid. The hexuronic acid was thought to be present in the colonic mucosa as a glycoprotein component and not as a connective tissue contaminant.

Kawaski et al[24] extracted glycoproteins from human colon with ethylene glycol. The major glycoprotein obtained contained as much as 80% carbohydrate, composed of glucosamine, galactosamine, galactose, fucose, sialic acid and ester sulphate. Threonine, serine and proline were the predominant amino acids. Immunofluorescent studies, using antisera against these glycoproteins were said to show that they were antigenically similar to glycoproteins in "intestinalised gastric mucosa" but were unreactive towards normal gastric glands.

The Involvement of Glycoproteins in Ulcerative Colitis

Changes in the patterns of epithelial glycoproteins associated with ulcerative colitis have been demonstrated by both chemical and histochemical methods. A detailed knowledge of the normal and pathological distribution of these different types of glycoproteins is an important prerequisite for any attempt to elucidate their biological significance. Although histochemical methods can be presumed to show the presence and location of some of these glycoproteins in a tissue, the exact chemical nature of such glycoproteins cannot be determined by histochemistry[23].

Histochemical Studies

The normal pattern of the epithelial glycoproteins in the colon and rectum of man, as revealed by histochemical

methods, has been described by several investigators [7,8,30-36]. There is general agreement that both sulphated and sialic acid containing glycoproteins are present in the colonic mucosa of man but there is some difference in opinion regarding the distribution of these materials between the surface epithelium, and the upper and lower halves of the crypts [36]. Changes in the pattern of the epithelial glycoproteins are associated with ulcerative colitis[7,8], but there is no general agreement as to the nature of these changes other than that there is a decrease in the quantity of glycoprotein present. Indeed in one study[8] the distribution of the colonic glycoproteins varied from case to case.

Chemical Investigations

Soergel et al[9] obtained rectal mucus from normal subjects and from patients with ulcerative colitis by irrigation of the rectum with hypertonic phosphate solution. Chemical analyses revealed the presence of fucose, galactose, hexosamine and sialic acid in material isolated from both normal and diseased colons. The carbohydrate composition appeared to remain constant, but the nitrogen content was 50 per cent higher in the samples isolated from diseased tissue than from normal tissue. Immunologic studies (double diffusion) showed that glycoproteins from normal human rectal

mucosa contained a variable number of serum proteins, particularly α and β globulin. These serum proteins were absent in mucus from ulcerative colitis patients. The authors postulated that these globulins might form an integral part of rectal mucus and that highly viscous α_2 - glycoproteins might contribute to the physical and chemical properties of the mucus.

Teague et al[10], in a study using mucosal biopsies and mucosal scrapings, reported the presence of two glycoproteins in colonic mucus. Both contained fucose, galactose, N-acetyl-glucosamine, N-acetyl-galactosamine and sialic acid. One however, contained mannose, whereas in the other mannose was a minor component. The amount of the mannose-rich component was significantly increased in ulcerative colitis.

The possibility that ulcerative colitis is an auto-immune disorder has been extensively investigated[5,37-42]. Broberger and Perlmann[38] found that the sera of 90% of children and 40% of the adults with ulcerative colitis contained an antibody that haemagglutinated with sheep red blood cells pre-coated with a phenol extract of sterile human colon. Fluorescein labelled gamma globulin from such patients was absorbed preferentially onto the epithelial cells of the crypts

of the colon[43]. Absorption was thought to be specific for sera from ulcerative colitis patients in that the antigen involved was immunologically unrelated to the ABH blood group substances. Further, fluorescent antibody labelling showed that sera from some patients with ulcerative colitis reacted with the cytoplasm of their own colonic epithelial cells[40]. On chemical analysis, the phenol extract of sterile human colon was found to contain galactose, glucose, mannose, fucose, xylose, hexosamine and traces of 3,6-dideoxyhexose, sialic acid and an acid hydrolyzable lipid. Thus, if ulcerative colitis is an autoimmune disease, it is possible that glycoproteins might play a central role in the antigenic stimulus[44].

Chemical changes in colonic epithelial glycoproteins have been associated with ulcerative colitis. Although these changes may be non-specific[37,45,46], a detailed knowledge of colonic epithelial glycoproteins in normal subjects and those with ulcerative colitis could contribute to a better understanding of the etiology and pathogenesis of the disease and may aid in its diagnosis. From the present literature, it appears that only crude glycoprotein extracts have been investigated, and as was pointed out above, they are probably contaminated with gastrointestinal secretions and the product of bacterial degradation.

The Involvement of Glycoproteins in Crohn's Disease

Crohn's disease is a chronic inflammation of the small and large intestine. Diffuse Crohn's disease of the large intestine is often misdiagnosed clinically, radiologically and histopathologically as ulcerative colitis.[5]

In a chemical analysis of the intestinal wall in a case of Crohn's disease of the ileum, Seppala et al[47] reported that the glycosaminoglycan content was higher in the diseased than in the healthy tissue, whereas the contents of total collagen, neutral-salt-soluble and acid-soluble collagen were the same in the disease and the healthy parts of the intestine. On electrophoresis, the glycosaminoglycans isolated from the diseased intestine always showed the presence of an extra, slow-moving, fraction which was similar to that found in glycosaminoglycans isolated from granulation tissue of rats. This fraction was thought to be glycoprotein in nature, but it was not further investigated.

To this degree therefore, chemical changes in glycoproteins have been implicated in Crohn's disease. No other study has been carried out to confirm this finding. Despite extensive investigations to reveal the clinical, radiological and pathological differences between ulcerative colitis and Crohn's disease, it is sometimes difficult to make a differential diagnosis[5]. It is hoped that a detailed knowledge of the

chemical differences, should they exist in the colonic glycoproteins in these disease entities, might aid in the differential diagnosis of these two diseases.

The Present Investigation

In this thesis, the author describes the isolation, fractionation and characterization of human colonic glycoproteins from purely epithelial sources. Epithelial glycoproteins were obtained from both normal and diseased human colons particularly those resected for ulcerative colitis and Crohn's disease.

In preliminary studies, rat colon was used as a model for experiments with human tissue. It was found that procedures suitable for rat colonic tissues could be successfully applied to a study of human colonic tissue.

MATERIALS AND METHODS

I. Preparation of samples for GLC and Colorimetric Analysis.

In general, samples for the various analyses were drawn from a stock aqueous solution of the glycoprotein of accurately known concentration (usually 2 mg glycoprotein per ml).

II. Gas Liquid Chromatographic Analysis.

Gas liquid chromatography (GLC) was carried out on a Hewlett Packard model No. 7610A high efficiency dual columan gas chromatography fitted with dual hydrogen flame ionization detectors and "on column injection". Helium was used as carrier gas. The following columns were used:

(a) 8' x 1/4" o.d.copper columns containing 10% S.E. 52 on 80 - 100 mesh diatoport S, operated isothermally at 190°C.

(b) 6' x 1/4" o.d. copper columns containing Chromosorb 103, operated isothermally at 145°C.

Peak areas were measured with a Hewlett Packard model No. 3370B electronic integrator. Analyses were carried out by an internal standard method using molar response factors determined in our laboratory as described by Reid et al[48].

A. Neutral Sugars

An accurately weighed sample (approximately 2 mg) of each freeze-dried material was hydrolyzed in a sealed glass tube at 100°C. for 36 to 50 hours according to the procedure of Lehnhardt and Winzler[49]. The free neutral sugars were analysed as their trimethylsilyl ethers (TMS-ethers) on column (a) by the method of Reid et al[48] using sorbitol as internal standard.

B. O-acetyl Groups

An accurately weighed sample of each of the lyophilized glycoproteins (2 mg) was treated with 0.1 M sodium methoxide in anhydrous methanol (o.5 ml.) at 4°C. for 0.5 hours[50]. The methyl acetate produced by transesterification was analysed on column (b) using benzene as the internal standard.

III. Colorimetric Analyses

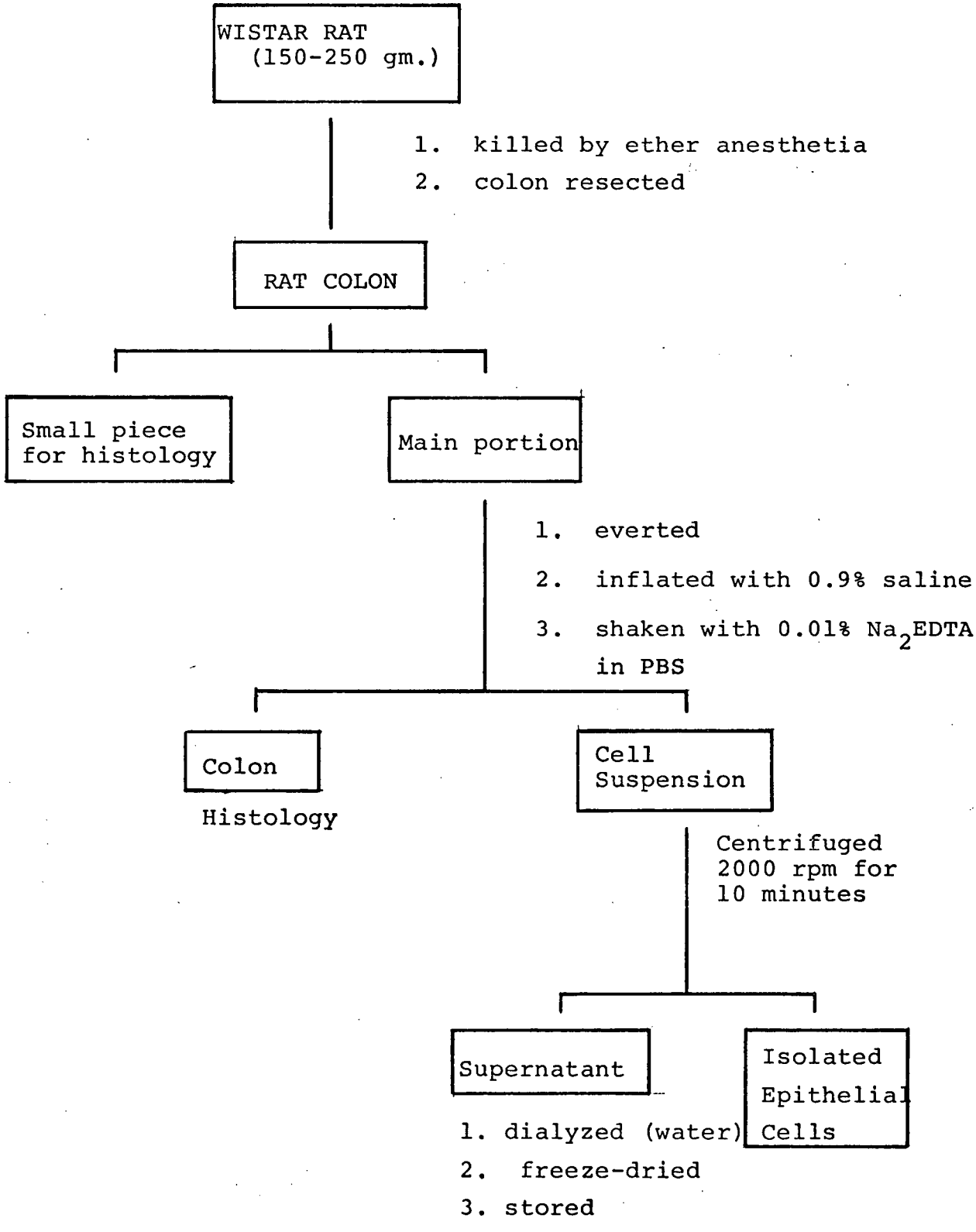
In most assays, various components of the glycoprotein other than the one under analysis were included as controls. Normally, these were galactose, fucose, N-acetylneuraminic acid and N-acetyl-glucosamine. On occassion, D-ribose, 2-deoxy-D-ribose, calf thymus DNA and yeast RNA were also used.

Hexoses were estimated by the phenol-sulphuric procedure of Dubois [51] using a mixture of galactose and fucose (10:3 w/w) as the standard. Fucose was estimated by the method of Dische and Shettles[3,52]. Protein was measured by the method of Lowry[53] with Hyland control serum (California, U.S.A.) as standard. Phosphorus was estimated by the procedure of Chen[54] and DNA by the method of Ceriotti[55] as modified by Short[56]. Hydrolysates of the glycoproteins prepared by the method of Lehnhardt and Winzler[49] were analyzed for hexosamine by modification [57] of the procedure of Winzler[58]. Total and bound sialic acids were estimated by the periodate resorcinol procedures of Jourdian et al[59], and free sialic acids by the method of Warren[60].

IV. Electrophoresis Studies

Cellulose acetate electrophoresis was carried out on 6" x 1" strips of Sepraphore III (Gelman Instrument Co.) using Tris barbital sodium barbital buffer (High Resolution Buffer, Gelman), pH 8.8, i= 0.1, for 30 minutes at 300 volts. Strips were stained with alcian blue [61] (pH 3.0). Cellulose acetate electrophoresis of rat glycoprotein samples was also carried out in 0.02 M pyridine HCl buffer, pH 5.5, and in 0.1 M pyridine formate buffer, pH 3.0.

Figure 1. Flow diagram of the method for the isolation of intact rat colonic epithelial cells.


WISTAR RAT
(150-250 gm.)
1. killed by ether anesthetia
2. colon resected
RAT COLON
Small piece for histology
Main portion
1. everted
2. inflated with 0.9% saline
3. shaken with 0.01% Na_2EDTA in PBS
Colon
Histology
Cell Suspension
Centrifuged 2000 rpm for 10 minutes
Supernatant
1. dialyzed (water)
2. freeze-dried
3. stored
Isolated Epithelial Cells

Agarose gel electrophoresis was performed with commercial agarose gel plates (Analytical Chemists Inc.) containing 1% agarose in Tris barbital sodium barbital buffer, pH 8.8, at a constant voltage of 90 volts for 30 minutes. The plates were stained with alcian blue, pH 3.0. Electrophoretic mobilities were measured relative to heparin.

V. Preliminary Experiments on the Isolation of Rat Colonic Epithelial Glycoproteins (Figures 1 - 3)

A. Isolation of Colonic Epithelial Cells

Intestinal epithelial cells were isolated by the procedure of Culling et al[62] (Figure 1). Wistar rats (150-250g) were killed with ether and the colons were removed. Epithelial cells were obtained by shaking the everted, inflated colons with 0.01% EDTA in PBS (phosphate-buffered saline), (8 g NaCl, 02.g KCl, 1.15g Na_2HPO_4 and 0.2 g KH_2PO_4 in 1 litre of water). The colons were alternately shaken for 5 minutes and incubated at 37°C for 5 minutes for a total period of one hour, the shaking medium being changed at 10 and 30 minutes. The pooled cell suspension was centrifuged at 2000 rpm for 10 minutes at 4°C. The residue, which contained epithelial cells, was washed twice with ice-cold PBS and recovered by centrifugation. The pooled supernatants were dialysed against distilled water, freeze dried and stored for future use.

Specimens (approximately 1/2 cm) were removed immediately after the colons had been everted and at the conclusion of the final shaking procedure. These were fixed in 10% formolcalcium, paraffin processed and cut at 5 μ. Sections were stained with Hematoxylin and Eosin (H & E) and with periodic acid/Schiff, alcian blue, pH 2.5[63]. Microscopic examinations of the stained sections enabled the degree of cell removal to be assessed.

Figure 2. Flow diagram of the method for the extraction of glycoproteins from rat colonic epithelial cells.

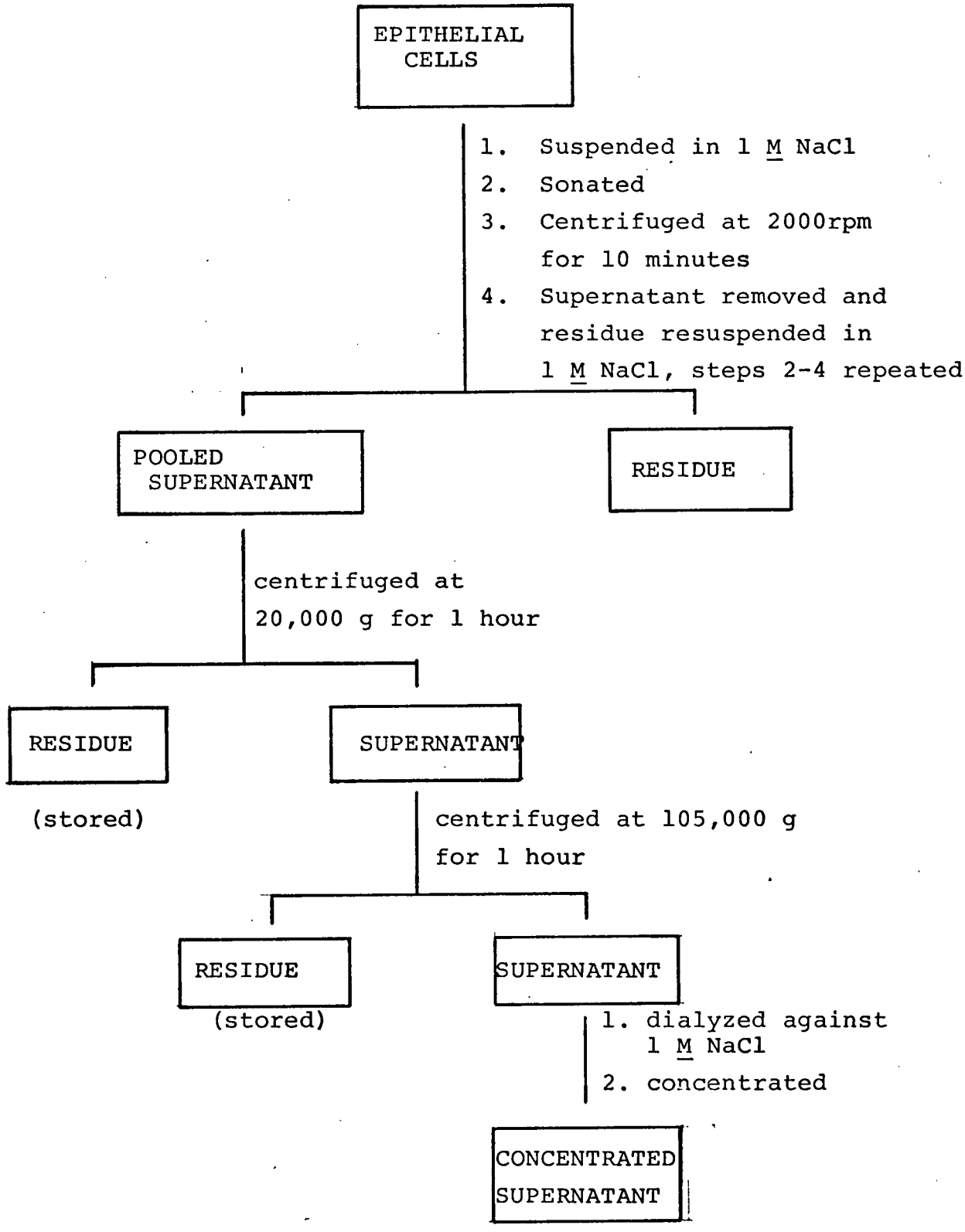
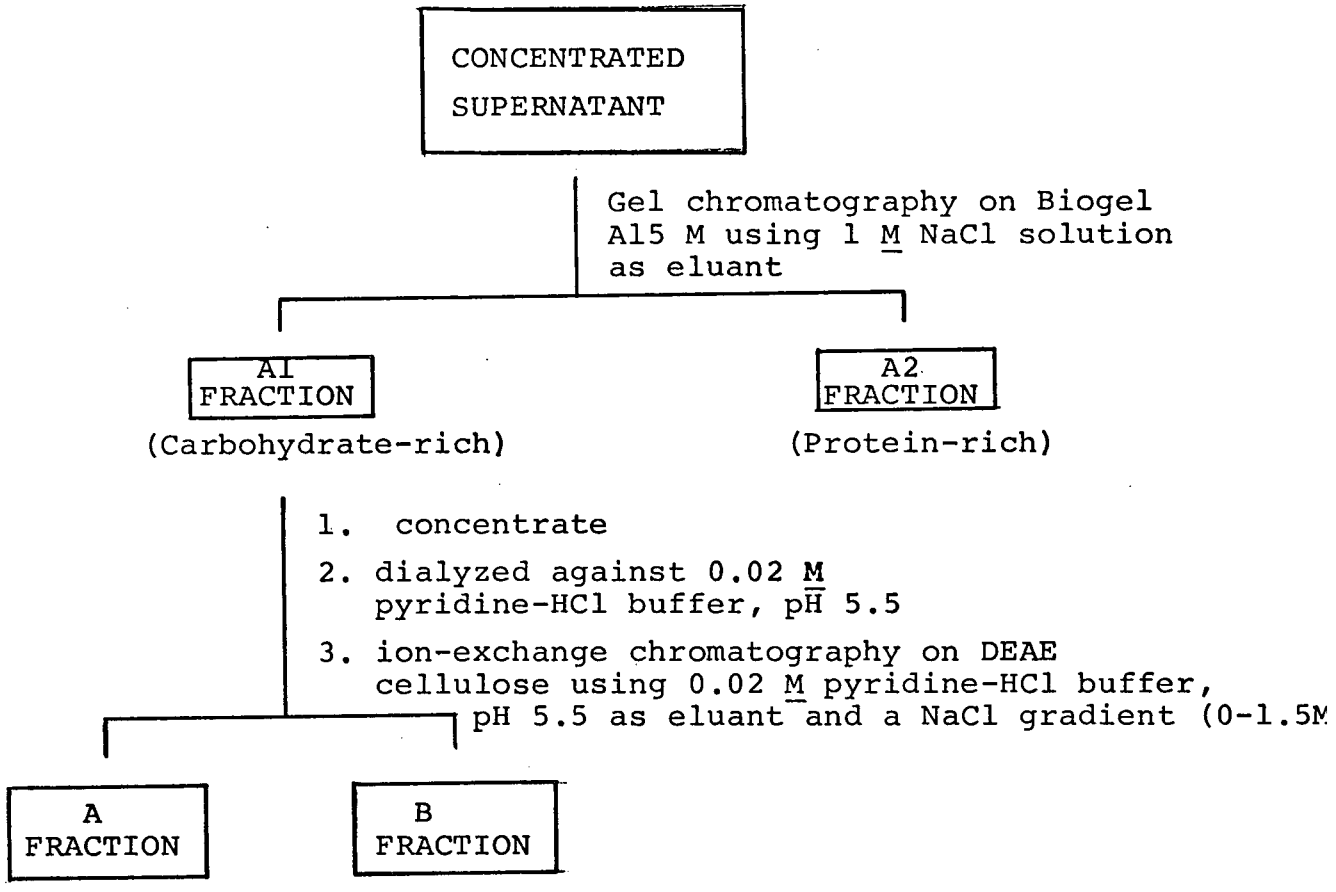

EPITHELIAL CELLS
1. Suspended in 1 M NaCl
2. Sonated
3. Centrifuged at 2000rpm for 10 minutes
4. Supernatant removed and residue resuspended in 1 M NaCl, steps 2-4 repeated
POOLED SUPERNATANT
RESIDUE
centrifuged at 20,000 g for 1 hour
RESIDUE
(stored)
SUPERNATANT
centrifuged at 105,000 g for 1 hour
RESIDUE
(stored)
SUPERNATANT
1. dialyzed against 1 M NaCl
2. concentrated
CONCENTRATED SUPERNATANT

Figure 3. Flow diagram of the method for the fractionation of Rat colonic epithelial glycoproteins.


CONCENTRATED SUPERNATANT
Gel chromatography on Biogel A15 M using 1 M NaCl solution as eluant
A1 FRACTION
(Carbohydrate-rich)
A2 FRACTION
(Protein-rich)
1. concentrate
2. dialyzed against 0.02 M pyridine-HCl buffer, pH 5.5
3. ion-exchange chromatography on DEAE cellulose using 0.02 M pyridine-HCl buffer, pH 5.5 as eluant and a NaCl gradient (0-1.5M
A FRACTION
B FRACTION

LEAF 24 OMITTED IN PAGE NUMBERING.

p. 24 does not exist

B. Extraction of Colonic Glycoproteins

The procedure for the extraction of colonic glycoproteins was a modification of a method previously described by Hakkinen[64] for the isolation of acid mucopolysaccharide (figure 2). A suspension of the colonic epithelial cells in ice cold 1M sodium chloride was sonated for periods of 30 seconds with a Biosonik Sonator (Bronswill Scientific) at settings of 1 and 80. The cell suspension was then centrifuged at 2000 rpm for 10 minutes and the residue was resuspended in cold 1 M sodium chloride solution and again sonated. This procedure was repeated until the quantity of residue obtained by centrifugation was constant. The pooled supernatants were centrifuged at 20,000 g for 1 hour and the residue was washed with 1 M sodium chloride solution and recentrifuged. The combined supernatants obtained were centrifuged at 105,000 g for 1 hour and the residue was washed in 1 M sodium chloride and recentrifuged. The final pooled supernatants were dialyzed against 1 M sodium chloride solution for 24 hours at $4^{o}C$ and concentrated to a convenient volume by means of an Amicon ultrafiltration apparatus with an XM 50 membrane, exclusion limit 50,000 M.W..

C. Fractionation of Colonic Glycoproteins

The fractionation of the concentrated 105,000 g supernatant is shown in figure 3. The concentrated supernatant

was applied to a column of Biogel A15M (100-200 mesh) (BIO-RAD Laboratories) and eluted with 1 M NaCl. The carbohydrate-rich fraction obtained was concentrated and dialyzed against 0.02 M pyridine-HCl buffer, pH 5.5 at 4°C for 24 hours. The retentate was applied to a column of DEAE-cellulose (DE22, Whatman), and was eluted with 0.02 M pyridine-HCl buffer, pH 5.5, containing a convex NaCl gradient (0 - 1.5 M). The various carbohydrate-rich fractions obtained were individually concentrated, dialyzed against distilled water overnight and recovered by lyophilization.

In preliminary experiments, the fractionation procedure was monitored for hexoses by the phenol-sulphuric method of Dubois et al[51], for protein by the Lowry procedure[53] and for sialic acid by the diphenylamine method of Werner and Odin[65]. In later experiments, the sialic acid assay was omitted.

VI Isolation of Human Colonic Epithelial Glycoprotein

A. Source of Specimens

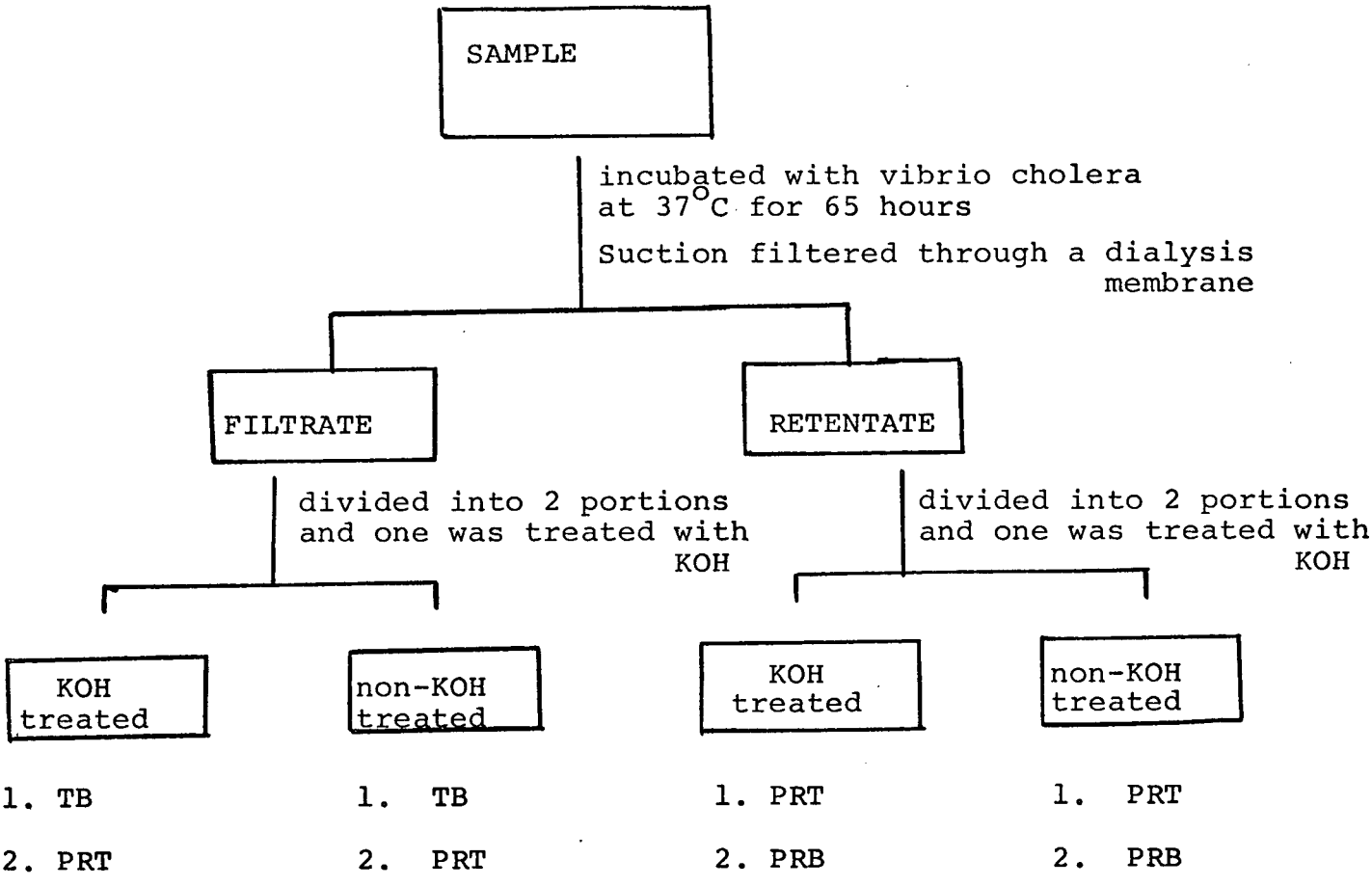
All specimens were obtained from the pathology department of the Vancouver General Hospital. They were transported to the laboratory in plastic bags packed in ice. The

length of time after surgery and between processing was from 1 to 3 hours. Cells were obtained from the following colon specimens: (a) the histologically normal parts of 20 specimens obtained from patients of both sexes and of ages ranging from 29 to 85,(average 53) and undergoing colectomy for carcinoma. The cells from these specimens were pooled and treated as one sample. (b) The entire colon from a 19 year old male cadaver which had been selected as kidney donor. (c) Surgical specimens from two cases of ulcerative colitis (two 60 year old female patients), and one case of ischemic colitis (39 year old female), one case of Crohn's disease of the large bowel and two cases of Crohn's disease of the terminal ileum (36 and 38 year old female patients). The cells from the two cases of ulcerative colitis were pooled as were those from the two cases of Crohn's disease of the terminal ileum.

B. Isolation of Human Colonic Epithelial Cells

The surgical specimens received from the pathology department at the Vancouver General Hospital had all been cut longitudinally along the length of the colon. After the fatty tissues had been removed from the serosal surface, the colons were sutured into their original tubular form with the mucosal surface on the outside. A cylindrical balloon, was inserted into each colon tube and was then inflated with isotonic saline.

Figure 4. Flow diagram of the procedure for the digestion studies of colonic epithelial glycoproteins of rat and human using vibrio cholera neuraminidase. Analyses for total (PRT) sialic acids by the periodate resorcinol method of Jourdian et al[59] were performed on samples of the retentate and the filtrate which were either (1) untreated or (2) treated with 0.1 N KOH for 1 hour at room temperature. Samples (both the KOH treated and non-KOH treated) of the retentate and the filtrate were further analyzed respectively for glycosidically bound sialic acids (PRB) by the periodate resorcinol method of Jourdian et al[59] and for free sialic acids (TB) by the thiobarbituric acid method of Warren[60].


SAMPLE
incubated with vibrio cholera at 37°C for 65 hours
Suction filtered through a dialysis membrane
FILTRATE
RETENTATE
divided into 2 portions and one was treated with KOH
divided into 2 portions and one was treated with KOH
KOH treated
non-KOH treated
KOH treated
non-KOH treated
1. TB
2. PRT
1. TB
2. PRT
1. PRT
2. PRB
1. PRT
2. PRB

Colonic epitehlial cells were removed from each specimen by shaking with 0.02% EDTA in PBS. The shaking and incubation procedures were identical to those employed with the rat colons (see figure 2). Histologic examinations, to monitor cell removal, was performed as described on page 21 .

C. Extraction and Fractionation of Human Colonic Epithelial Glycoproteins

The procedures employed were similar to those described with rat colons (see pages 22 to 24).

VII. Saponification Studies

A known weight of each freeze dried glycoprotein (o.2 mg/mg) was treated with 0.1 N. potassium hydroxide for 2 hours at room temperature. Aliquots (0.2 to 0.5 ml) were withdrawn at 0, 0.5, 1 and 2 hours and after neutralization with the appropriate quantity of 1N sulphuric acid were analyzed for sialic acid by the periodate resorcinol method of Jourdian et al[59].

VIII. Digestion Studies

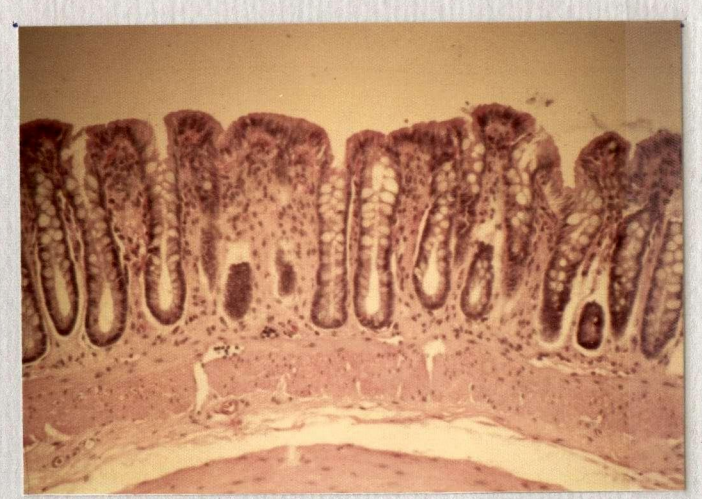
These were carried out as shown in figure 4. An accurately weighed sample (5 to 12 mg) of each freeze dried glycoprotein was dissolved in 0.05 M sodium acetate buffer,

pH 5.5 (Containing 2 X 10^{-2}% sodium azide and 1 X 10^{-1}% calcium chloride) and incubated with Vibrio cholera neuraminidase (100 units / ml incubate) (Behringwerke) for 24 hours at 37°C. A further 100 units of enzyme per ml incubate was then added and incubation was continued for a further 41 hours. The incubate was vacuum filtered through a dialysis sac with an exclusion limit of approximately 100,000 MW.. The retentate and dialysate was obtained were freeze dried and redissolved in a known volume of distilled water. Analyses for total (PRT) sialic acids by the periodate resorcinol method of Jourdian et al[59] were performed on samples of the retentate and the filtrate which were either (1) untreated or (2) treated with 0.1 N KOH for 1 hour at room temperature. Samples (both the KOH treated and non-KOH treated) of the retentate and the filtrate were further analyzed respectively for glycosidically bound sialic acids (PRB) by the periodate resorcinol method of Jourdian et al[59] and for free sialic acids (TB) by the thiobarbituric acid method of Warren[60].

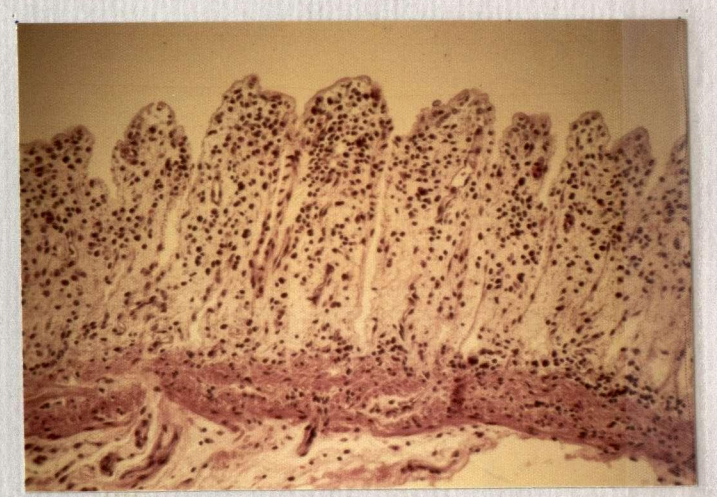
IX. Immunodiffusion Studies.

These were carried out on commercially available Ouchterlony plates (Hyland, California, U.S.A.).

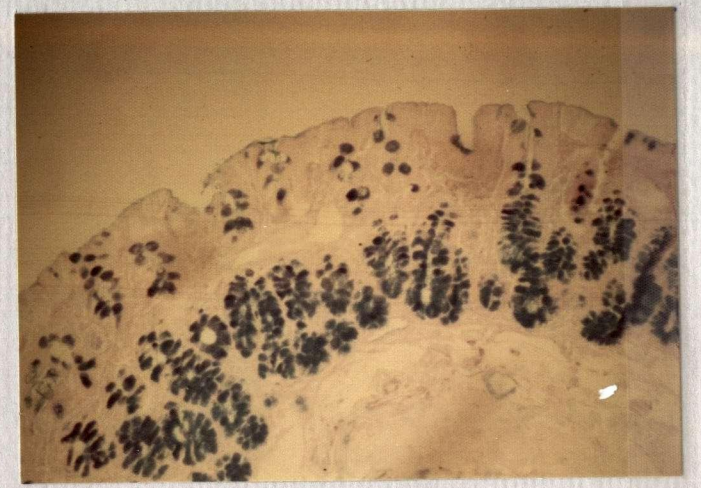
Figure 5. Photomicrographs of sections of human colon obtained from a specimen resected for carcinoma of the colon. The tissue was taken (A) before and (B) after treatment with 0.02% (0.54 mM) EDTA in phosphate buffered saline. In this case, complete removal of epithelial mucus secreting cells in (B1) and (B2) was seen and there was no apparent damage to the basement membrane. Sections (A1) and (B1) were stained with alcian blue - PAS technique and sections (A2) and (B2) were stained by H & E.



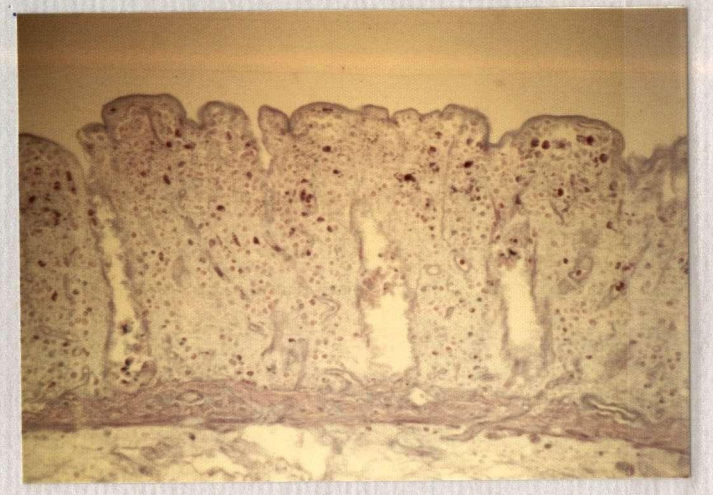
A 2



B 2



A 1



B 1

X. Preparation of Antisera

A modification of the method of Kopp et al [66] was followed in the preparation of antisera against rat colonic epithelial glycoprotein. 7.7 mg of freeze dried glycoprotein (fraction A) was suspended in PBS, pH 7.3, at a concentration of 10 mg per ml and was mixed with an equal volume of complete Freunds adjuvant (DIFCO Lab.) by squirting into a shot glass. The mixture was then injected into the four foot pads of a 3 lb. female New Zealand rabbit. Three weeks later, a booster dose of the same strength was injected subcutaneously. After a further 2 weeks, the rabbit was given a subcutaneous injection of a suspension of 6.6 mg of rat glycoprotein in 1.4 ml of a 1:1 mixture of PBS and Pertusis Vaccine (Connaught Medical Research Laboratories). Two weeks after the last injection, 5 ml of blood was drawn from the marginal vein of the rabbit ear. Serum was obtained by centrifuging the clotted blood at 3000 rpm for 30 minutes.

RESULTS AND DISCUSSION

1. Isolation of Epithelial Cells

Complete cell removal was accomplished in all rat experiments when inflated and everted rat colons were shaken with 0.01% (0.27 mM) EDTA in PBS. The majority of the cells were removed during the first 30 minutes of shaking. The epithelial cell suspension was washed twice with PBS and the cells were recovered by centrifugation. The average packed cell volume obtained was 1.16 $\pm$ 0.24 ml of cells per rat colon. When this isolation procedure was applied to specimens of human colon, the use of 0.02% (0.54 mM) EDTA was necessary to achieve removal of the epithelial cells. In 40% of the specimens, complete removal of cells was accomplished (figure 5), while in the others, from 10 to 90 % of the cells were removed. The cells at the base of the crypts proved to be the most difficult to remove.

2. Extraction of Colonic Epithelial Glycoproteins

The isolated cells were suspended in 1 M NaCl, and sonated in an ice-water bath. Normally, complete rupture of all

the colonic epithelial cells required 5 to 6 periods of sonation of 30 seconds duration each. Sonation was judged to be complete by the occurrence of a constant residue after centrifugation at 2000 rpm for 10 minutes. Under the light microscope, this residue appeared to be composed of cell membrane fragments. The sonate was centrifuged at 105,000 g and the supernatant obtained was usually concentrated, to one-tenth of its volume by Amicon ultrafiltration membrane (exclusion limit 50,000 M.W.). Recovery of the glycoproteins was usually greater than 90% (see page 37).

The efficiency of the sonation procedure was compared with two other currently used homogenization methods. Known volumes of packed cells were homogenized with (a) a motor-driven Pestle OMNI-MIXER, (b) a pressure homogenizer and (c) sonation. Each homogenate obtained was centrifuged successively at 2000 rpm, 20,000 g and 105,000 g. The 105,000 supernatant and the 3 residues from each procedure were analyzed for protein by the Lowry procedure[53] and for hexose by the phenol-sulphuric method of Dubois et al[51]. The results obtained indicated that sonation gave a more complete extraction of the carbohydrate rich materials from the cells, and that this could be accomplished in a much shorter time. Furthermore, spectrophotometric estimation of the DNA in the 105,000 g supernatants indicated that the supernatant from the sonation procedure contained the least quantity of DNA.

TABLE 1. Percentage recovery of hexose and sialic acid after concentration of the 105,000 g supernatant of intestinal epithelial glycoprotein by the various procedures

Method	Hexose recovery (%)	Sialic acid recovery (%)
Sephadex G-25	67.3 [5]	67.3 [3]
Amicon ultra-filtration	83.8 [4]	85.8 [1]
Hollow fibre (BIO-RAD)	33.0 [1]	0 [1]

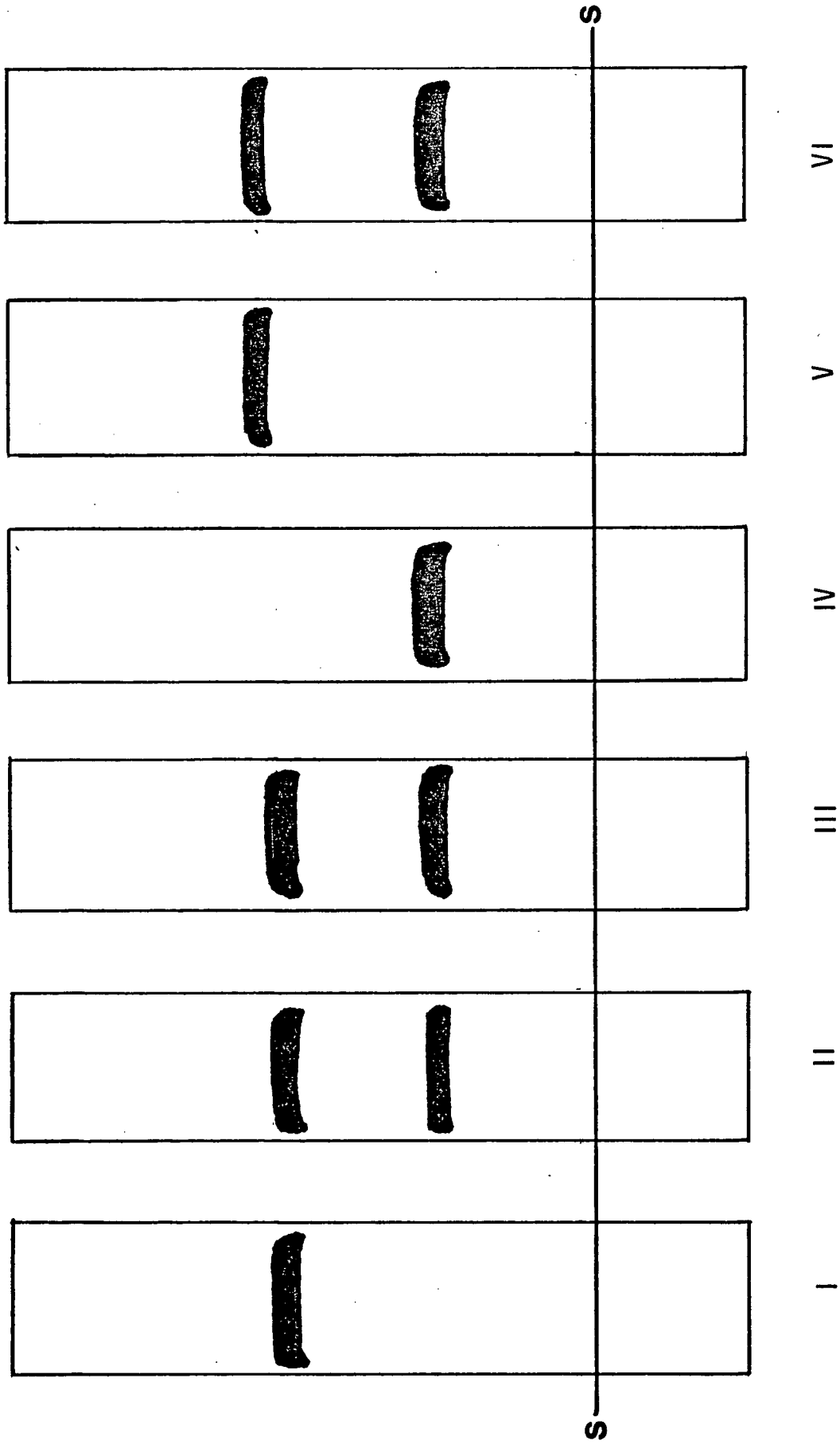
Note: (a) The number in the bracket represents the number of experiments done to arrive at the above averages.

(b) The degree of concentration in each case was about 8 to 10 times.

(c) Hexose was determined by the phenol-sulphuric procedure of Dubois[51] and sialic acid was estimated by the diphenylamine method of Werner and Odin[65].

(3) The exclusion limit of the membrane used in Amicon ultra-filtration was 10,000 (PM10).

Figure 6. Monitoring of the fractionation of rat colonic epithelial glycoproteins by cellulose acetate electrophoresis at pH 8.8. All strips were stained with alcian blue at pH 3. I, herapin standard; II, 105,000g supernatant; III, fraction A1; IV, fraction A; V, fraction B; VI, fractions A & B combined.


S
VI
V
IV
III
II
I
S

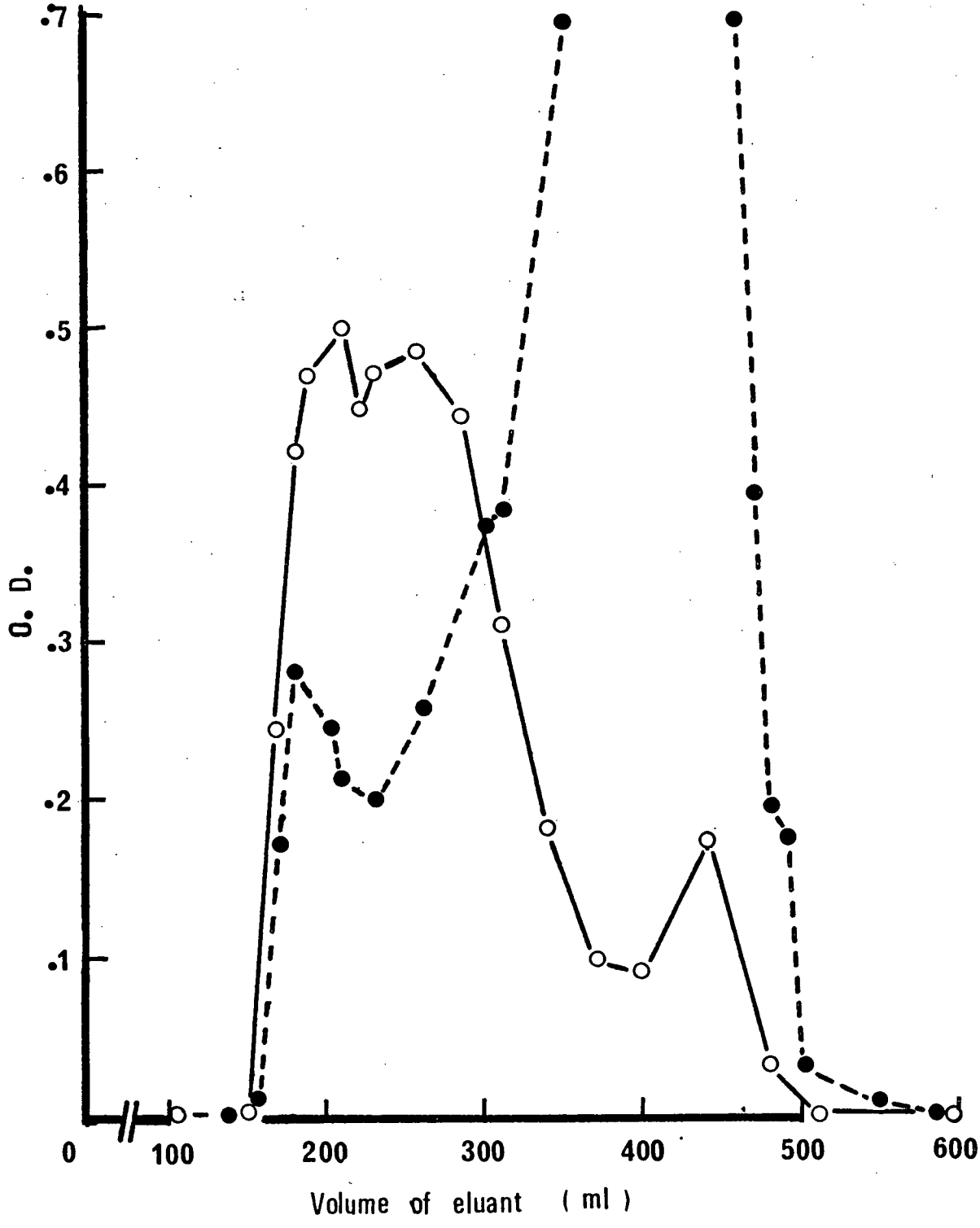
During the preparation of the glycoproteins, it was necessary to concentrate solutions on at least three occassions. It was desirable therefore, to choose a concentration procedure which caused the smallest losses of glycoprotein. For this reason, three currently used methods of concentration were investigated: namely, (a) Sephadex G-25 (coarse), (b) Amicon ultrafiltration and (c) hollow fibre concentration dialysis. The results of this study, shown in Table 1, indicated that Amicon ultrafiltration led to the smallest loss of glycoprotein as judged by the recovery of sialic acid and hexose. By this method, the average recovery was more than 85% of the starting material. A further 5 to 10% of the total glycoprotein could be recovered, in some cases, by rinsing the membrane with 1 M NaCl solution. This procedure which utilizes membranes with size-limiting pores (ultrafiltration), has been claimed to be the most selective method for concentrating biological materials and to be the least injurious to protein solutes [67]. Thus, Amicon ultrafiltration was employed in all of the concentration and most of the dialysis processes used in this work.

3. Fractionation of Colonic Epithelial Glycoproteins

A. Preliminary Model Experiments with Rat Glycoproteins

Electrophoretic examination of the 105,000 g supernatant at pH 8.8 indicated the presence of two components

Figure 7. Agarose A15M column chromatography of the 105,000 g concentrated supernatant extracted from the isolated colonic epithelial cells of a human sample resected for carcinoma of the colon. Column size 83X 2.5 cm.. Fractions were eluted with 1 M NaCl. Hexose (O) and protein (●) were determined by the procedures of Dubois[51] and Lowry[53] respectively. A carbohydrate-rich fraction A1 and a protein-rich fraction A2 were obtained.


.7
.6
.5
.4
.3
.2
.1
O. D.
0
100
200
300
400
500
600
Volume of eluant (ml)

which stained with alcian blue at pH 3.0. The mobility of the faster moving band was similar to that of the heparin standard and was approximately twice as fast as that of the slower moving band (see figure 6).

Fractionation of this supernatant by gel chromatography on Biogel A15M using 1 M NaCl as eluant yielded a carbohydrate-rich fraction A1 and a carbohydrate-poor but protein-rich fraction A2. An immunodiffusion study revealed that fraction A2 reacted with antirat plasma protein prepared in rabbits while fraction A1 didnot. Presumably, fraction A2 contained plasma proteins and other low molecular weight substances. Fraction A1 was concentrated, dialyzed against 0.02 M pyridine HCl buffer, pH 5.5, and was then fractionated by ion exchange chromatography on DEAE cellulose using 0.02 M pyridine HCl buffer, pH 5.5, containing a convex salt gradient (0 to 1.5 M NaCl) as eluant. Two carbohydrate-rich peaks were obtained which were designated fractions A and B according to their order of elution. Cellulose acetate electrophoresis showed that fraction B corresponded to the fast moving band in the 105,000 g supernatant while fraction A represented the slower moving band (figure 6). This fractionation procedure was repeated on two new samples of the 105,000 g supernatant of rat colonic epithelial cells, and similar results were obtained.

Figure 8. DEAE cellulose ion-exchange column chromatography of the carbohydrate-rich fraction A1 obtained from Agarose A15M column chromatography of human colonic cell sap (105,000g supernatant) obtained from a sample resected for carcinoma of the colon. Column size 83 X 2.5 cm.. Fractions were eluted with 0.02 M pyridine HCl buffer, pH 5.5, containing a convex NaCl gradient (0-1.5 M). Two distinct peaks were obtained (fractions A and B). Hexose was estimated by the procedure of Dubois[51] and protein measured by the method of Lowry[53]. (O), hexose; (●), protein and (••••) the salt gradient.

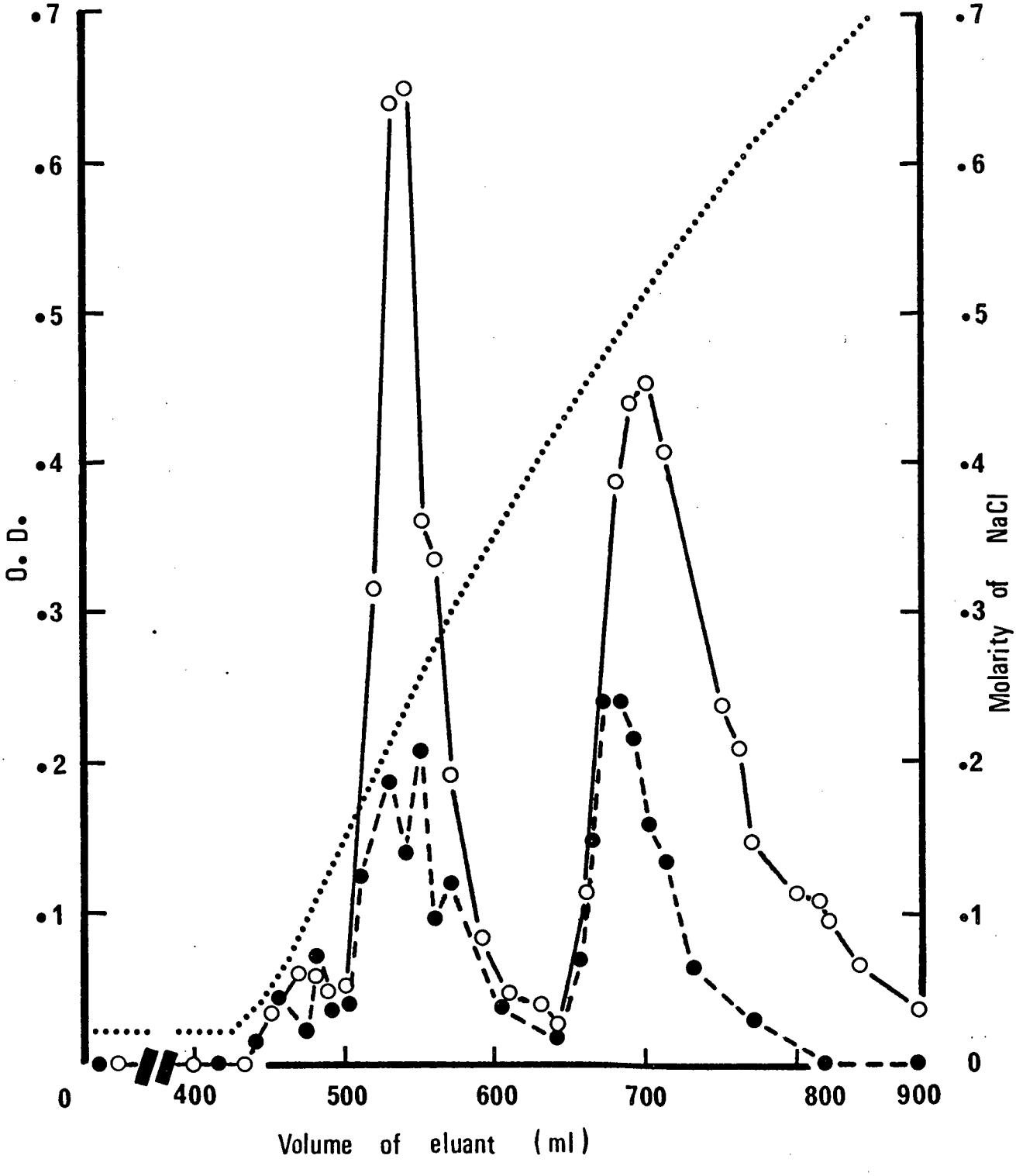

O. D.
Molarity of NaCl
Volume of eluant (ml)
0
400
500
600
700
800
900
•1
•2
•3
•4
•5
•6
•7

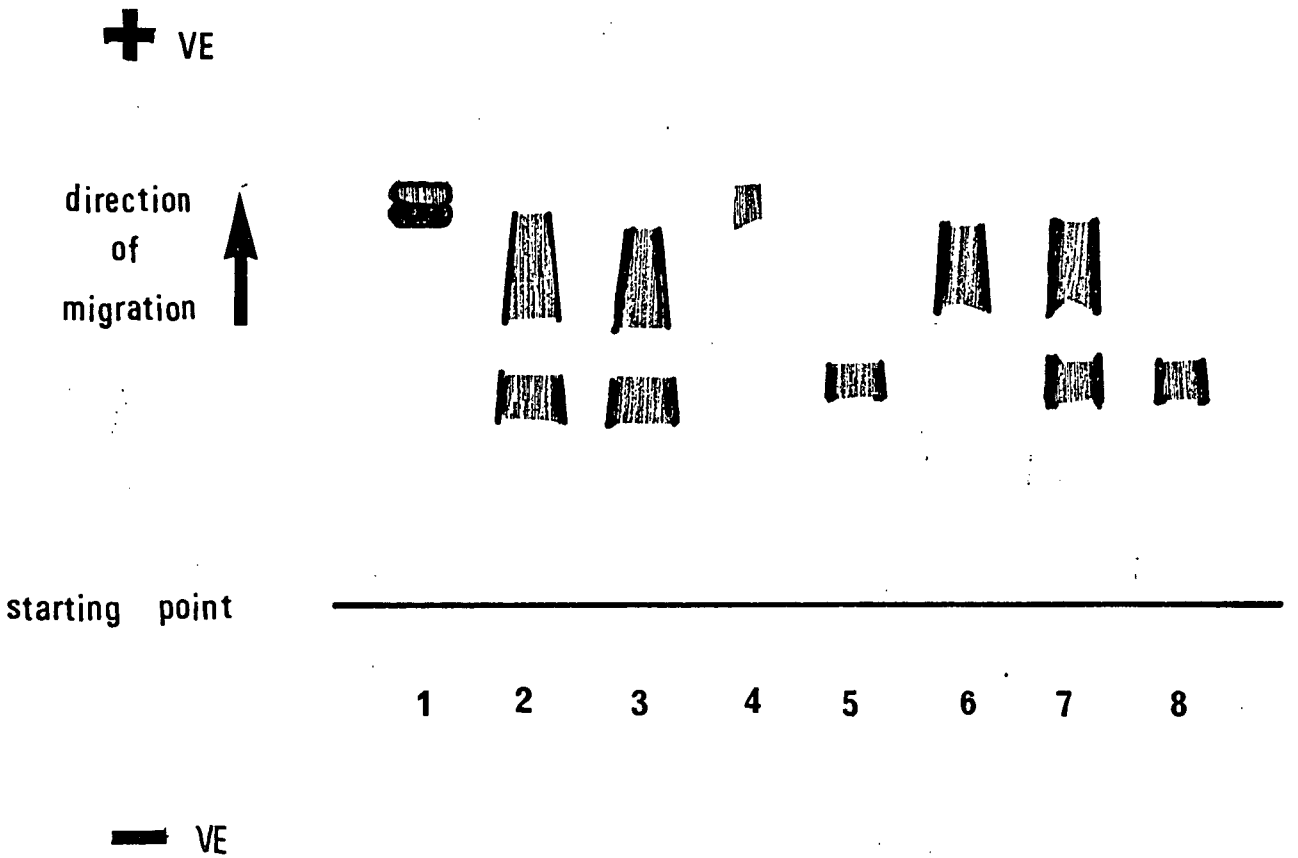
TABLE 2. Agarose gel electrophoretic mobilities of the sample obtained from the various stages in the fractionation of colonic epithelial glycoproteins extracted from normal and diseased human colons.

	SAMPLES					
	Normal (Cancer)	Normal	Ischemic Colitis	Crohn's (colon)	Crohn's (ileum)	Ulcerative Colit
Heparin	1.0	1.0	1.0	1.0	1.0	1.0
105,000g Supernatant	0.29	0.34	0.35	0.22	0.20	0.34
	0.79	0.82	0.79	0.89	0.83	0.87
Fraction A1 (carbohydrate-rich)	0.29	0.35	0.36	0.19	0.25	0.37
	0.74	0.78	0.87	0.91	0.88	0.85
Fraction A2	1.0	1.01[1]	0.98[2]	1.05	1.03[3]	1.01
First DEAE fraction (fraction A)	0.35	0.35	0.35	0.20	0.24	0.37
Second DEAE fraction (fraction B)	0.82	0.84	0.84[4]	0.87	0.87	0.85
Calf thymus DNA control (streak)		0 to 0.88	0 to 0.89	0 to 0.9	0 to 0.91	0 to 0.58

Note: (1) (2) and (3), very faint traces of alcian blue staining with mobilities of 0.45, 0.41 and 0.3 respectively were found.

(4) very faint trace of alcian blue positive material with mobility of 0.34 was observed.

Figure 9. Illustration of a typical agarose gel electrophoresis pattern of the various fractions in the fractionation of the 105,000g supernatant of a human sample. In this case, the 105,000g supernatant was extracted from the isolated colonic epithelial cells of a sample resected for carcinoma of the colon. Heparin was used as the standard. Electrophoresis was performed with commercial agarose plate containing 1% agarose in Tris barbital sodium barbital buffer, pH 8.8, at a constant voltage of 90 volts for 30 minutes. The plates were stained with Alcian blue, pH 3. 1, heparin; 2, 105,000g supernatant; 3, fraction A1, 4, fraction A2; 5, fraction A; 6, fraction B; 7, fractions A and B combined.


+ VE
direction of migration
starting point
1
2
3
4
5
6
7
8
- VE

B. Experiments on Human Colonic Epithelial Cells

Similar procedures to those developed on the rat model were applied to the extraction of glycoproteins from human colonic epithelial cells. Fractionation of the 105,000 g supernatant of the 1 M NaCl extract of these cells on Biogel A15M using 1 M NaCl as eluant yielded a carbohydrate-rich fraction A1 and a protein-rich fraction A2. (figure 7) Fraction A1 was dialyzed against 0.02 M pyridine HCl, pH 5.5, and was fractionated by ion exchange chromatography on DEAE cellulose using 0.02 M pyridine HCl, pH 5.5, containing a convex NaCl gradient (0 - 1.5 M) as eluant. Two apparently homogenous fractions (fractions A and B) were obtained from each normal and diseased sample (figure 8) except in one case of Crohn's disease of the terminal iteum where multiple peaks were observed in Fraction B.

The results of agarose gel electrophoresis are shown in table 2. All the 105,000 g supernatants were found to show two alcian blue staining bands (figure 9). One of these had a mobility similar to that of heparin while the other migrated at a much slower rate. Fraction A1, obtained by Biogel A15M fractionation, was found to contain both alcian blue positive bands. DEAE cellulose chromatography of A1 separated the two bands into fractions A and B. Fraction B was found to have a mobility similar to heparin and approximately twice that of Fraction A. All fractions B showed similar

TABLE 3. Chemical Analyses of fractions B.
(All values are expressed in ug/mg of freeze dried sample).

	Protein	Phosphorus	DNA	Hexose	GLC Hexose fucose	Sialic acid
RAT	84	74.0	1080	245	0	16
NORMAL (Cancer)	122.5	67.4	1030	235	0	15
NORMAL	123.5	67.9	1090	250	0	17
ULCERATIVE COLITIS	11.9	76.8	1090	250	0	19
CROHN'S (Colon)	16.6	77.9	1180	251	0	16
CROHN'S (Ileum)	22.4	66.8	920	-	0	19
ISCHEMIC COLITIS	131.8	47.3	690	-	0	35
CALF THYMUS DNA (CONTROL)	18.3	66.8	1000	210	-	11
RNA (CONTROL)	27.4	76.8	0	340	-	2

Chemical analyses were performed by the following procedures: hexose by the method of Dubois[51], protein by the procedure of Lowry[53], phosphorus by method of Chen[54], DNA by the indole procedure of Ceriotti[55] as modified by Short[56] and sialic acid by the periodate resorcinol procedure of Jourdian[59]. Other than the rat and the two nucleic acid controls, all the samples were obtained from human colons.

TABLE 4. Molar ratio of sugars in colonic epithelial glycoproteins (fraction A)

	Molar Ratios of Sugar			
	Fucose	Galactose	Hexosamine	Sialic acid
RAT	1	3.35	2.2	1.45
NORMAL (Cancer)	1	3.15	3.7	2.7
NORMAL	1	2.9	2.6	1.7
ULCERATIVE COLITIS	1	2.1	2.1	1.5
CROHN'S (colon)	1	1.35	1.2	0.32
CROHN'S (ileum)	1	5.3	2.1	3.73
ISCHEMIC COLITIS	1	3.4	3.35	3.3

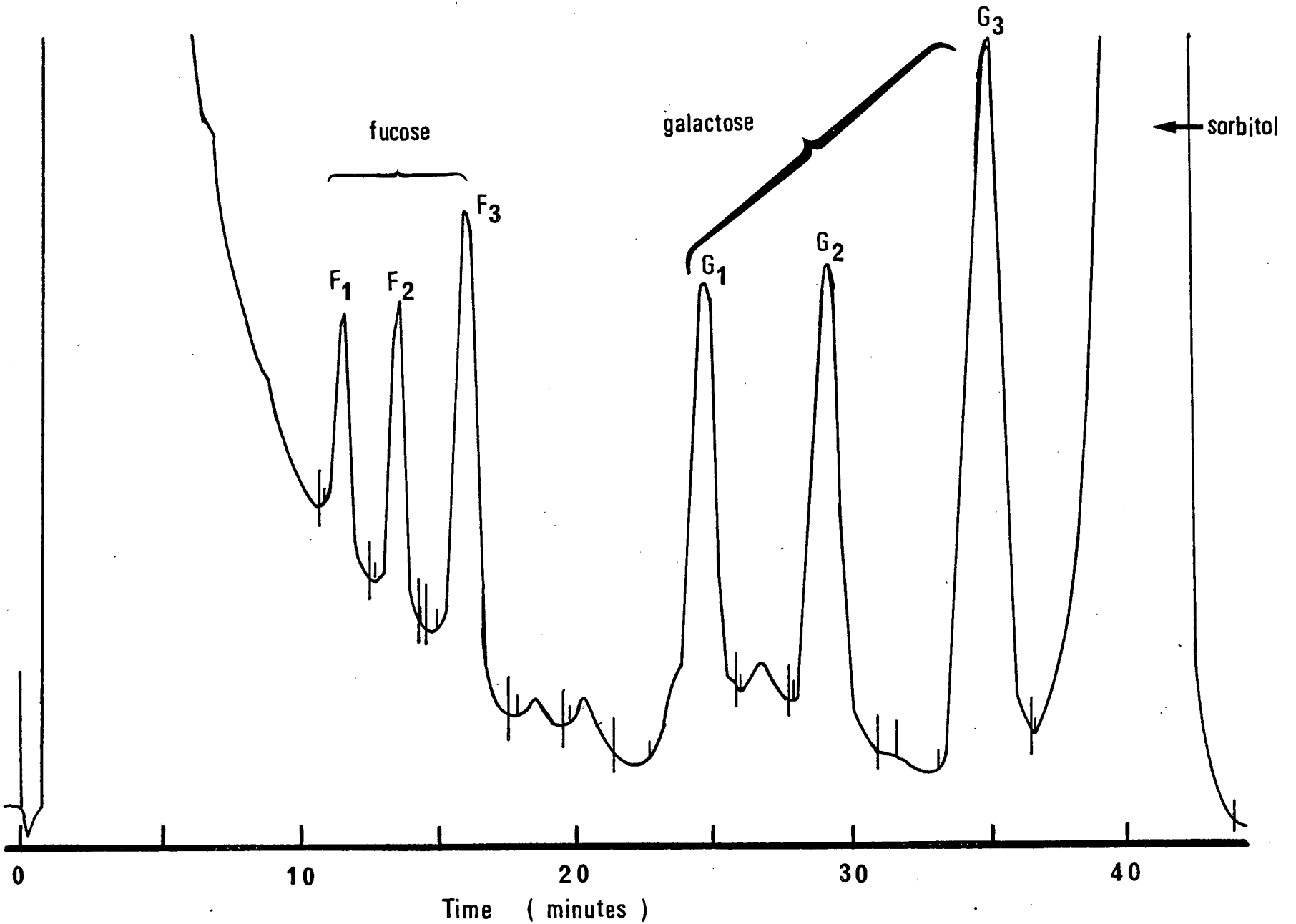
Note: Glycoprotein samples were treated with Dowex $50H^+$ in 0.01 HCl at $100^{\circ}C$ for 36 to 50 hours and the Hydrolysates were analysed for fucose and galactose by GLC and for hexosamine by a modification of a procedure of Winzler[57,58]. Sialic acid values represented the total sialic acid obtained from a periodate-resorcinol assay of the fully saponified glycoprotein samples.

TABLE 5. Chemical analyses of the colonic epithelial glycoproteins (fraction A). All values are expressed in ug per mg of freeze dried sample.

	Protein	Hexose	Phosphorus	Fucose
RAT	84.3	230	0.2	60.1
NORMAL (Cancer)	58.5	170	0	41.3
NORMAL	65.0	195	0.2	57.6
ULCERATIVE COLITIS	82.8	140	0.2	41.2
CROHN'S (Colon)	67.9	105	0.2	46.2
CROHN'S (Ileum)	116.0	-	0.1	-
ISCHEMIC COLITIS	53.7	-	0.1	-

Chemical analyses were performed by the following procedures: protein by the procedure of Lowry[53], hexose by the phenol-sulphuric procedure of Dubois[51], phosphorus by the method of Chen [54] and fucose by the procedure of Dische and Shettles[4,52]. (-) indicates that the analysis was not performed.

Figure 10. GLC of the trimethylsilyl ethers of the neutral sugars of colonic epithelial glycoprotein. The sample illustrated in this typical chromatogram was obtained from the histologically normal part of surgical carcinoma specimens obtained from patients undergoing colectomy. Only fucose and galactose were detected and each of the two sugars had resolved into three peaks (1), (2) and (3).


fucose
galactose
sorbitol
F1
F2
F3
G1
G2
G3
0
10
20
30
40
Time (minutes)

electrophoretic mobilities. In the case of fractions A, the two samples obtained from specimens resected for Crohn's disease moved much slower than fractions from other sources.

4. Blood Contamination

The cells isolated from some human samples were contaminated by blood. In view of this, the sonation and fractionation procedures described above were carried out on samples of whole human blood. Blood components had a similar eluted volume to the included second peak (see above). Thus, none of the glycoproteins examined below appeared to arise from contamination with blood proteins.

5. Analyses of Fractions B

The results obtained from an analysis of fractions B are shown in table 3, in which is included for comparative purposes similar analyses of calf thymus DNA and yeast RNA. All these fractions absorbed strongly at 260mμ, failed to migrate on electrophoresis at pH 3.0 and contained no detectable fucose, galactose or hexosamine. Based upon their high phosphorus content, the results of the Indole assay[56] and the similarity of the analytical results to calf thymus DNA, fractions B are probably DNA.

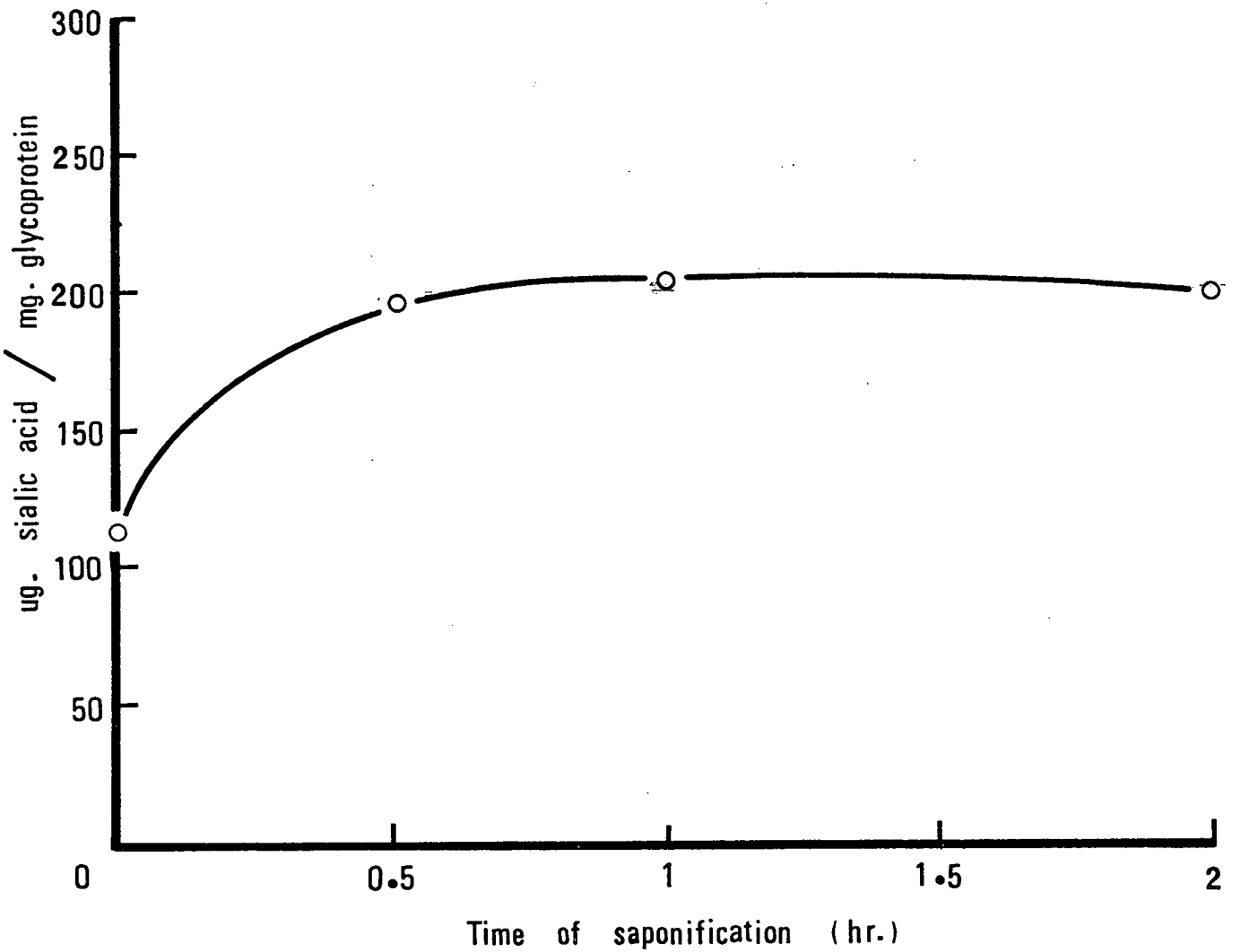
The small quantity of sialic acid found in fractions B using the periodate-resorcinal assay is probably due to interference in this assay by large quantities of DNA [56]. The result of the DNA test (indole) indicates that it is unlikely that RNA was present in any of these fractions. The low DNA values given by the sample with Ischaemic colitis was probably due to the presence of a contaminant. It is probable that this contamination produced the faint alcian blue positive band (in agarose gel electrophoresis) associated with the second DEAE fraction of this sample (see table 2).

Of interest is the Lowry protein analysis. 12% Lowry protein was found in all human samples except for the three obtained from colons resected for ulcerative colitis, and Crohn's disease, where only 1 to 2% Lowry protein was detected. This low protein value was very similar to that found in the reference standard, calf thymus DNA, the protein of which had been removed by exhaustive dialysis. There is insufficient data in this study to draw any conclusion on the significance of these differences in Lowry protein values.

6. Analyses of Fractions A

The results obtained from analyses of these fractions are shown in tables 4 and 5. All the fractions contained fucose, hexose, hexosamine, protein and sialic acid. Gas chromatographic analysis of the hydrolyzates indicated that

Figure 11. Illustration of a typical saponification time curve in which a sample of human colonic epithelial glycoprotein, obtained from a surgical specimen resected for carcinoma of the colon, was treated with 0.5% KOH. The amount of total sialic acid present was estimated at 0.5, 1 and 2 hour by the periodate resorcinol method of Jourdian [59]. Saponifications appeared to be completed at 0.5 hours.


300
250
200
150
100
50
0
ug. sialic acid / mg. glycoprotein
0.5
1
1.5
2
Time of saponification (hr.)

galactose was the only hexose present (figure 10). The molar ratio of the sugars in each sample is shown in table 4. Insignificant quantities of phosphorus were detected.

7. Saponification Studies

When untreated and KOH treated samples of the same colonic epithelial glycoprotein were tested for total sialic acid by the periodate resorcinal procedure (PRT) of Jourdian et al[59] the amount of sialic acid detected was found to be greater in the fully saponified sample. This implied that some sialic acids which were undetectable by PRT in the natural state were rendered detectable under alkaline conditions. It has been reported that the periodate-resorcinol procedure for the estimation of total sialic acid depends upon the reaction of the resorcinal reagent with an aldehyde group, produced by periodate oxidation, at position C7 of the sialic acid side chain [59,68]. Substituents present at either C7 and or C8 of the side chain will therefore prevent the clevage of the bond between C7 and C8 by periodate oxidation, leaving the sialic acid unreactive to PRT. Consequently, the removal of these substituent groups chemically (for example, treatment with KOH) will render the sialic acids reactive to PRT. This can be used to explain the discrepancy in the amount of sialic acid detected in the unsaponified and fully saponified samples ofthe same colonic epithelial glycoprotein. Substituents which are alkalie-labile must be present at C7 and or C8 of

TABLE 6. Percentage of side chain substitution in the sialic acids of colonic epithelial glycoproteins.

Source of Sample	Percentage side chain substitution in sialic acids
NORMAL (Cancer)	49.2
NORMAL	25.0
ULCERATIVE COLITIS	55.4
CROHN'S (Colon)	41.0
CROHN'S (Ileum)	53.1
ISCHEMIC COLITIS	61.0

Note: Colonic epithelial glycoproteins were saponified in 0.5%KOH for 120 minutes. Samples of the fully saponified and the unsaponifiec glycoprotein were tested for total sialic acid by the periodate resorcinol procedure of Jourdian (59). The percentage of side chain substitution in sialic acid is given by the following expressior

$$\text{Percentage of side chain substitution} = 100 \times \frac{PRT_{(\text{sap. sample})} - PRT_{(\text{unsap. sample})}}{PRT_{(\text{sap. sample})}}$$

where PRT= periodate resorcinol estimate of total sialic acid.

some of the sialic acid side chains of this glycoprotein. Furthermore, analysis (GLC) of the methyl acetate produced by treatment of the glycoprotein with sodium methoxide in methanol indicated that alkaline-labile O-acetyl groups were present in these glycoproteins.

In order to obtain a true estimate of the sialic acids present in the glycoproteins under study, it is necessary to remove all the substituent groups by alkaline treatment (saponification) before the PRT test is applied. To find the optimal time for the alkaline treatment procedure, each colonic epithelial was subjected to a saponification time study. A typical saponification time curve is shown in figure 11. For the glycoproteins studied, saponification was complete between 30 and 60 minutes. As a result, 60 minutes was chosen as the optimum time for all subsequent saponification procedure. Thus, for a particular glycoprotein, the total amount of sialic acid present is estimated by the PRT value of a fully saponified sample. The difference in PRT values of the fully saponified and the unsaponified glycoprotein isa measure of the amount of sialic acid substituted at either C7 or C8. Consequently, the percentage of sialic acid having side-chain substitution at C7 or C8 can be calculated. The results obtained with the various colonic glycoproteins under study are tabulated in table 6. As compared with the normals, an increase in the percentage of side chain substitution of sialic acids was found in all the diseased colons. However,

more normal colons must be analysed before conclusions can be drawn.

8. Digestion Study of Vibrio Cholera Neuraminidase Digest

In an independent experiment carried out in our laboratory, a sample of rat colonic epithelial glycoprotein was incubated with vibrio cholera neuraminidase for 48 hours. The amount of free sialic acid released by the enzyme was estimated by the thiobarbituric acid procedure of Warren[60]. The results revealed that less than 30% of the total sialic acid present in the glycoprotein was released. Failure of the enzyme to bring about total release of the sialic acid from the glycoprotein could be attributed to the presence of substituent groups such as O-acetyl groups at C4 of the sialic acid, for it is generally believed that sialic acids substituted at C4 are resistant to vibrio cholera neuraminidase digestion [69]. If this were true, then removal of these substituents should increase the susceptibility of the glycoprotein to enzyme digestion. Indeed, when a sample of a fully saponified rat colonic epithelial glycoprotein was treated with vibrio cholera neuraminidase for 53 hours, about 95% of the sialic acids were released as free sialic acid. Thus, it was concluded that sialic acids substituted at C4 were present in rat colonic glycoproteins and that saponification was necessary to bring about total liberation of N-acetylneuraminic acid by neuraminidase.

Substitution of the sialic acids by alkaline-labile groups is not however confined solely to position C4. As was discussed in the section on saponification, the colonic glycoproteins under study have some sialic acids with O-acetyl groups at position C7 and or C8 of the polyhydroxy side chain.

The digestion study outlined in figure 4 was designed to permit a partial estimation of the percentage substitution occurring at each of positions C4, C7 and C8 on the sialic acid residue. After exhaustive digestion of the glycoprotein with vibrio cholera neuraminidase, the incubate was vaccuum filtered through a membrane with an exclusion limit of approximately 100,000 M.W.. Sialic acid released by the enzyme would pass through the membrane and be found as free sialic acid in the filtrate. As sialic acids substituted at C4 are resistant to vibrio cholera neuraminidase digestion, these free sialic acids would not be expected to contain any substitutents at C4. On the other hand, sialic acids found in the retentate would still be bound to the glycoprotein and would therefore be substituted at C4. Quantitatively, the amount of free sialic present in the filtrate as estimated by the thiobarbituric acid test of Warren for free sialic acid (TB) would represent the amount of sialic acid residues free of substitutents at C4. On the other hand, the amount of sialic acid containing substitutents at C4 would be given by the periodate resorcinol test (PRB) for bound sialic acid on the retentate. The sum of the free and bound sialic acids

TABLE 7. Substitution patterns of sialic acids in colonic epithelial glycoproteins.

	% of substitution occurring at positions			
	C_O	C_7 or C_8	C_4	C_4+C_7 or C_8
NORMAL (Cancer)	34.0	16.3	18.6	31.1
NORMAL	58.1	19.8	16.8	5.3
ULCERATIVE COLITIS	19.0	12.0	23.4	45.6
CROHN'S (Colon)	38.3	9.2	22.8	29.7
ISCHEMIC COLITIS	17.2	10.6	21.7	50.4

Note; sample for Crohn's disease of the ileum was not available for this analysis.

would give a total amount of sialic acids present. Hence, the percentage of C4 substitution occurring in the sialic acid residue of a glycoprotein can be calculated.

Saponified and unsaponified samples of both the filtrate and retentate were analyzed for total sialic acid by the periodate-resorcinol procedure of Jourdian et al[59]. The degree of substitution at C7 or C8 of the filtrate and the retentate was then calculated (for discussion see previous sections on saponification). For the filtrate (containing sialic acids unsubstituted at C4), two types of sialic acids were found. These were estimated as follows: (a) the PRT value for the non-KOH treated sample would represent the amount of unsubstituted sialic acids (unsubstituted at C4, C7 and C8) (b) the difference in PRT values of the KOH treated and non-KOH treated samples would indicate the amount of sialic acids substituted at C7 and or C8. In the retentate, two types of sialic acids substituted at C4 were found and estimated as follows: (a) the PRT value of the non-KOH treated retentate sample indicates the amount of sialic acids with no substitutions at C7 or C8. (b) the difference in PRT values of the KOH treated and non-KOH treated samples would indicate the amount of sialic acids substituted at C4, C7 and or C8.

With the procedures employed in this study, it is not possible to differentiate between substitutents at C7 and C8. Neither can the procedures be utilized to estimate substitutents at C9. The results of this digestion study, performed on human colonic epithelial glycoproteins obtained from normal and diseased colons, are as shown in table 7. The sialic acid substitution of colonic glycoproteins obtained from diseased colons appears to be different from the controls. Since the results are based on single specimens the results must be interpreted with a large degree of caution.

9. GLC Analysis of O-acetyl Groups

The number of O-acetyl groups present in the colonic epithelial glycoprotein was estimated by GLC as the methyl acetate after trans-esterification with 0.1 M sodium methoxide in anhydrous methanol. O-acetyl groups were found in all the samples but the results were inconsistent, and it was not possible to obtain quantitative data.

GENERAL DISCUSSION

Fractions A

Fractions A glycoproteins appear to be homogeneous by DEAE chromatography, agarose gel electrophoresis at pH 8.8 and cellulose acetate electrophoresis at pH 8.8. Chemical analysis of these glycoproteins reveal the presence of fucose, galactose, hexosamine, sialic acid, protein and little or no phosphorus. Qualitatively, they resemble glycoproteins isolated from gastric juice[70], colonic mucosa[11,16,17,24], small intestine mucosa [11,17,19,21]and rectal mucus [9], and they are apparently uncontaminated with nucleic acid. Since the glycoproteins were extracted from washed epithelial cell preparations they should be reasonably free of contaminants such as connective tissue components and materials arising from the faecal stream. Further, since proteolytic digestion was avoided, and relatively mild fractionation techniques were employed, it is possible that an undegraded glycoprotein was obtained. There appears to be no other report in the literature of the isolation and characterization of colonic glycoproteins from a purely epithelial source.

The data presented in table 4 shows: (a) that there was a difference in the molar ratio of the sugars of the glycoprotein isolated from the normal and diseased human colons, (b)

that in each of the glycoprotein studied, the four different types of sialic acids found, (that is: (i) unsubstituted or C9 substituted sialic acids (ii) sialic acids substituted at C4 and or C9, (iii) sialic acids substituted at C7 and or C8, (iv) sialic acids substituted at C4 and at C7 and or C8) were present in different quantities (see table 7). It is tempting to conclude that these differences are related to changes associated with the disease processes but there is clearly insufficient data available to support such a conclusion.

Under normal conditions, variations of the chemical compositions of the colonic glycoproteins might occur in the following manner; (a) along the length of the colon. (b) from the bottom to the top of the crypts of Lieberkuhn. Thus, the differences found in this study could be attributed to the different anatomical region of the colon from which the specimens were obtained rather than to the disease process. Also, in some experiments not all the cells were removed, with those at the base of the crypts the most difficult to remove. It has been reported that colonic goblet cells mature as they migrate from the bottom to the top of the crypts[71]. Failure to remove the cells in the lower half of the crypts in some experiments may therfore have excluded the majority of young cells.

In addition to the above, microheterogeneity can occur in the normal biosynthesis of colonic epithelial glycoproteins. Despite extensive investigation on the biosynthesis of intestinal glycoproteins[3], the detailed mechanism remains unknown. In general, glycoproteins are synthesized in colonic epithelial cells by the following mechanism[3,4]. The peptide chain is synthesized in the rough endoplasmic recticulum according the to Orthodox RNA template theory. Sugars are added in a step-wise sequence as monosaccharide units. The enzyme attachment of the first sugar via a sugar nucleotide transferase proceeds while the peptide is still attached to the polyribosomes or shortly after their separation. The remainder of the oligosaccharide chain is completed inthe smooth membranes and in the golgi apparatus. The glycosyl transferases are specific for both the acceptor and the donor. The order of the sugar sequence depends on the kinetic sequence of the transferases involved. For example, sialytransferases require a high molecular weight acceptor and this explains the terminal position of sialic acid in an oligosaccharide chain. In spite of the non-randomness in the synthesis of the oligosaccharide chain as well as the specificity of the transferases, this mechanism of synthesis, unlike the RNA-directed synthesis of proteins, may introduce microheterogeneity as a result of (a) the production of incomplete oligosaccharide chains and (b) the occurrence of incorrect monosaccharide sequence. The results in this work may therefore represent microheterogeneity of one or both of these types.

TABLE 8. Molar ratios of sugar found in colonic epithelial glycoproteins expressed as percentages.

	FUCOSE	GALACTOSE	HEXOSAMINE	SIALIC ACID
NORMAL (Cancer)	9.5	29.9	35.0	25.6
NORMAL	12.2	35.4	31.7	20.7
ULCERATIVE COLITIS	14.9	31.3	31.3	22.4
CROHN'S (Colon)	25.8	34.8	31.0	8.3
CROHN'S (Ileum)	8.2	43.7	17.3	30.6
ISCHEMIC COLITIS	9.1	30.8	30.3	29.9

Note: Glycoprotein samples were treated with Dowex $50H^+$ in 0.01 N HCl at 80^0C. for 36 to 50 hours and the hydrolysates were analysed for fucose and galactose by GLC and for hexosamine by a modification [57] of a procedure of Winzler[58]. Sialic acids were estimated by the periodate resorcinol procedure of Jourdian [59] for total sialic acid.

The following discussion is very tentative therefore, and it is realized that the differences discussed may be a result of normal variations and be entirely unrelated to the disease process. Under pathological conditions, changes in the chemical composition of the colonic epithelial glycoproteins found in this study might be ascribed to one or both of two general classes of changes (a) alterations in the normal biosynthesis of the glycoproteins at different stages of the synthesis and, (b) alterations in the normal degradation of the glycoprotein after synthesis is complete.

In this study, a general increase in the proportion of C4 substituted sialic acids was found in glycoproteins isolated from all diseased samples (table 7). It is now generally believed that sialic acids substituted at C4 are resistant to vibrio cholera neuramindase digestion[79]. In a pathological environment, constant removal of non-C4-substituted sialic acids might occur resulting in the presence of a high proportion of C4-substituted sialic acids in the glycoprotein pool. Thus, these could be called "residual" glycoproteins. On the other hand, the increase in C4-substituted sialic acids may also be the result of the production of "adaptive" glycoproteins in the epithelial cells. That is, the glycoproteins synthesized are capable of surviving the pathological environment. A detailed kinetic study on the biosynthesis of intestinal glycoprotein in normal and in pathological conditions would provide information on whether or not such "residual" or "adaptive"

glycoproteins are involved in these disease processes.

When the molar ratios of the sugars of the glycoproteins (fucose, galactose, hexosamine and sialic acid) were expressed as percentage values (table 8) it was found that the amount of galactose and hexosamines were quite constant in the glycoproteins obtained from the different diseased tissues of the colon. However, the amount of sialic acids and fucose were found to vary from disease to disease. Of particular interest was the low percentage of sialic acids found in the glycoprotein obtained from Crohn's disease of the large intestine. This low percentage value does not explain the slower mobility of the glycoprotein on agarose gel electrophoresis (table 2), since the glycoprotein obtained from Crohn's disease of the terminal ileum which contained three to four times more sialic acid, exhibited a similar slow electrophoretic mobility. This low mobility however, was not found in glycoproteins obtained from ulcerative colitis (table 2). The above finding, if confirmed, might aid in the differential diagnosis of ulcerative colitis and Crohn's disease of the colon.

It is interesting to note that the percentage of non-substituted sialic acid dropped from 58% in the normal to about 35% in glycoproteins from patients with Crohn's disease and from patients with carcinoma and to about 18% in both ulcerative colitis and ischaemic colitis. This reduction is counterbalanced

by a corresponding increase in the percentages of sialic acids substituted at C4 or at C7 and or C8. In general, the percentage of side chain substitution was elevated in all of the diseases studied and suprisingly inthe tissues resected for carcinoma. In view of the high risk of malignant change associated with ulcerative colitis[72] and the increasing number of reports of carcinoma complicating Crohn's disease [5], this finding, if confirmed, might contribute to an understanding of the etiology and pathogenesis of these diseases.

Teague et al[10] reported the presence of two glycoproteins in human colonic mucosa. One of these, excluded from Sepharose 2B, was mannose free; the other which was of molecular weight, and which contained significant quantities of mannose. Since mucosal scrapings were the source of glycoprotein, contamination of the preparations by serum proteins is probable. These would be expected to appear in the included fraction obtained from Sepharose 2B and contribute mannose to this fraction. It is surprising therefore that serum proteins could not be detected by immunological methods in either of the Sepharose fractions obtained by Teague et al[10]. Traces of mannose were found to be present in the glycoproteins obtained from "mucous plugs" by Johansen[26], and in the glycopeptides isolated by proteolytic digestion of minced colon by Sky-Peck et al[23]. However, the presence of mannose in intestinal glycoproteins has not been reported by other workers.[11,16,17,19-21,24]

The analytical methods employed in this investigation will enable the detection of a quantity of mannose as low as 2% by weight of the galactose present. (that is, approximately 0.2% of the dry weight of the glycoprotein). Mannose could not be detected in any of the glycoproteins isolated in this study but it is possible that a mannose containing glycoprotein was present in the low molecular weight 'A2 fraction' obtained from the agarose A15M fractionation. This fraction probably corresponds to the included Sepharose 2B fraction investigated by Teague et al[10] and it is therefore of interest that the 'A2' fractions tested in this work contained serum proteins.

Further Work Proposed

As was mentioned previously (see page 59), colonic epithelial cells progress through a maturation cycle as they migrate from the bottom to the top of the crypts [71]. In order to assess whether there is a significant variation in the chemical composition of the glycoproteins obtained from colonic cells at different phases of their maturation cycle, epithelial cells at different levels of the crypt should be isolated. Removal of cells from different levels of the crypts in rat small intestine had been reported by Weimer[73]. Furthermore, it is also necessary to investigate any variations in the glycoproteins along the whole length of the colon.

Once the positional variations if any are known, purified glycoproteins should be obtained from normal and diseased colons. Chemical analyses of these should be carried out to test the reproducibility of the present data. In addition, the amount of O-acetyl groups and the percentage of each type of amino acid present in these glycoproteins should be ascertained and the structure of the oligosaccharide units should be elucidated. The results from these investigations would permit a much better comparison of the glycoproteins present in the normal andin the various pathological conditions.

Once purified glycoproteins from delineated areas of each normal and diseased colonic epithelium have been obtained these should be used in the preparation of antisera. Such antisera would allow an assessment of the distribution (or cross reactivity) of these glycoproteins in the gastrointestinal tract, and in addition, would facilitate the investigation of whether or not the antigenic determinants present in the glycoproteins are disease specified.

In view of the difficulties experienced in obtaining human samples, miniaturization of the fractionation and chemical procedures would enable investigations to be carried out on small samples such as biopsy specimens. Also, since blocks of formol-calcium fixed, paraffin processed tissues of diseased tissues are readily available, the successful application of such

micro techniques to the investigation of these tissues would allow an extensive investigation on readily available material.

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APPENDIX

Since the completion of the experimental work reported in this thesis, an independent study carried out in this laboratory has found that the estimate of the quantity of sialic acids substituted at C_7 and or C_8 is in error.

In the PRT assay, the sample under test is first treated with periodic acid. Sialic acids unsubstituted at C_7 and C_8 are susceptible to periodate oxidation resulting in the formation of carbonyl groups at C_7. These carbonyl groups react with the resorcinol reagent forming a chromogen, with an absorption maxima at 630 m . Sialic acids substituted at C_7 and or C_8 are resistant to periodate oxidation and it was assumed therefore, that they were unreactive towards the resorcinol reagent.Recently however, it has been shown that the resorcinol reagent reacts with the C_7 and or C_8 substituted sialic acids (PRS reaction). This reaction gives a significant O.D. reading at 630 mμ , thereby raising the PRT reading attributed to sialic acids unsubstituted at C_7 and C_8. Thus, the O.D. reading at 630 m is no longer a true estimation of C_7 unsubstituted sialic acids and values reported in tables 6 and 7 need to be recalculated. It should be noted however, that the PRT values for KOH treated samples need not be corrected as none of the sialic acids contain substitutents at C_7 and would all participate in the PRT reaction. The extinction coefficient of the PRS reaction is 0.2798 times

that of the PRT assay. In recalculating the data as reported in tables 6 and 7, the following equator was employed.

$$PRT_{(actual)} = \frac{PRT_{(observed,\ non\text{-}KOH)} - 0.2798\ PRT_{(KOH)}}{0.7202}$$

The recalculating data are as shown in tables A and B. A comparison of the original and revised calculations suggests that the original interpretation of the data still holds true.

TABLE A. Percentage side chain substitution in the sialic acids of colonic epithelial glycoproteins.

Source of Sample	Percentage side chain substitution in sialic acids
NORMAL (Cancer)	65.9
NORMAL	34.9
ULCERATIVE COLITIS	80.0
CROHN'S (Colon)	44.0
CROHN's (Ileum)	73.4
ISCHEMIC COLITIS	84.7

TABLE B. Substitution patterns of sialic acids in colonic epithelial glycoproteins.

	% of substitution occuring at positions			
	C_o	C7orC8	C4	C4+C7orC8
NORMAL (Cancer)	27.7	22.6	6.5	43.2
NORMAL	50.4	27.5	14.7	7.4
ULCERATIVE COLITIS	14.3	16.7	5.7	63.3
CROHN'S (Colon)	34.7	12.8	11.3	31.2
ISCHEMIC COLITIS	13.1	14.7	2.1	70.0