Purification, Properties, and Analysis of Human Asthmatic Bronchial Mucin†

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ABSTRACT: A high molecular weight, mucin-type glycoprotein has been isolated from a sample of human bronchial secretion obtained from an asthmatic patient. The glycoprotein elutes in the void volume of a Sepharose 4B column, and its mobility is unchanged in the presence of dithiothreitol. Examination of the material in the analytical ultracentrifuge under equilibrium conditions gave an estimated minimal molecular weight of 1.8×10^6 with aggregation to 10×10^6 or greater. Analysis showed the predominant amino acids to be serine, threonine, and proline with a low content of methionine and cysteine; glucosamine and galactosamine were present in approximately equimolar amounts and comprised 28% by weight of the glycoprotein. Composition analysis after alkaline borohydride treatment showed that the saccharide chains were O-glycosidically linked through *N*-acetylgalactosamine to both serine and threonine residues in the peptide backbone. Carbohydrate analysis by gas–liquid chromatography identified galactose, fucose, glucosamine, galactosamine, and sialic acid in an approximate molar ratio of 3:3:2:2:1. The sialic acid is present as *N*-acetylneuraminic acid. A portion (7%) of the saccharides are present as galactosyl→*N*-acetylgalactosaminyl residues linked to the protein core. A glycopeptide fraction was isolated following pronase digestion and had a molecular weight of 1.5×10^5. This value was not significantly changed by either removal of sialic acid or exposure to guanidinium chloride. These data support the presence of large clusters of oligosaccharides which are covalently linked to the serine and threonine residues of the peptide.

Human mucosal epithelia from all sources are coated with mucus. The specific properties of mucus vary among organ tracts (respiratory, salivary, gastric, and cervical), but the overall chemical and physical similarities reflect a common lubricative and protective function. The respiratory mucus has the additional specific roles of particulate clearance and maintenance of proper water balance in the tracheobronchial tract.

The chemical composition of unfractionated human bronchial secretions from healthy and diseased states has been generally defined as to total inorganic, protein, lipid, and carbohydrate content (Basch et al., 1941; Chernick & Barbero, 1959; Matthews et al., 1963; Masson et al., 1965; Schultze & Heremans, 1966). Physical properties, such as viscosity and elasticity, have also been examined (Denton, 1960).

An understanding of the relationship between the macromolecular components of respiratory mucus and the physiological functions of this secretory product requires, in part, isolation and characterization of the individual glycoprotein components.

In general, fractionation of mucus by gel exclusion chromatography on Sephadex G-200 or Sepharose 4B produces two major peaks. The high molecular weight excluded component, commonly referred to as mucin or epithelial glycoprotein, is carbohydrate rich and is apparently responsible for the physicochemical structure and rheological properties of the mucus secretion (Havez et al., 1967). The majority of studies on the glycoprotein component of bronchial secretion have been histochemical and autoradiographic (Havez et al., 1967; Lamb & Reid, 1969; Sturgess & Reid, 1972). The chemical composition of some preparations has been described (Werner, 1953; Havez et al., 1968; Roussel et al., 1972, 1975; Lamblin et al., 1973; Roberts, 1974; Boat et al., 1976; Lafitte et al., 1977). Bhattacharyya and Lynn have reported on the saccharide structure of a glycoprotein isolated from the alveoli of patients with alveolar proteinosis (Bhattacharyya & Lynn, 1977; Bhattacharyya et al., 1976). The carbohydrate in this macromolecule, however, is of the asparagine-linked, mannose-containing type and appears unrelated to the predominant mucin glycoprotein. This paper deals with the purification, characterization, and partial structural identification of the major macromolecular component of bronchial secretion. There are considerable technical difficulties in collecting adequate amounts of normal human bronchial secretion. Accordingly, the high molecular weight glycoprotein component discussed in this report was isolated from the tracheobronchial secretions of an asthmatic patient. Separate studies in our laboratory have demonstrated that organ cultures of human trachea produce a mucin glycoprotein with physical and chemical properties similar to if not identical with those described for the bronchial secretion product. In addition, preliminary analyses of aspirate mucus from pediatric patients show the presence of comparable components. Thus, this study should provide background data for comparison with the structural features of bronchial mucins obtained from healthy individuals and from individuals with defined pulmonary pathology. It should be noted that mucus from patients with cystic fibrosis has been extensively investigated but cannot be considered representative of normal material (Roussel et al., 1975).

Experimental Procedures

Materials. Human bronchial secretion obtained by a 4-day collection (100 mL) from an asthmatic patient was generously provided by Dr. William Lynn, Department of Biochemistry, Duke University. The patient was a secretor and the crude mucus sample had blood group A activity (Morgan & Watkins, 1951). The crude mucin was examined for microbial contamination by culturing an aliquot on growth medium. No microorganisms were observed from this sample or subsequent

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collections from the same patient.

The secretion was promptly frozen and maintained in the frozen state until workup. The material was thawed, diluted 1 to 10 with H_2O, and adjusted to 5 mM NaN_3 to prevent microbial contamination during subsequent procedures. The diluted sample was stirred overnight at 4 °C and centrifuged at 7000*g* for 20 min to remove a small amount of cellular debris. There was no visible gel-like material present in the pellet. The pellet contained less than 10% by weight of the starting material was not examined further. The supernatant, which contained over 90% of the carbohydrate content of the original material, was applied to a 5 × 92 cm column of Sepharose 4B (Pharmacia Fine Chemicals). Chromatography was performed at pH 5.0 as indicated in Figure 1. The high molecular weight void volume component, identified as the mucin glycoprotein, was dialyzed against H_2O at 4 °C and lyophilized (150 mg).

Analytical Methods. Analyses for the carbohydrate components of glycoproteins were carried out as follows: chromatographic column effluents were screened for neutral sugar by the phenol–sulfuric acid method (Dubois et al., 1956) with galactose as a standard, and for sialic acid by the thiobarbiturate procedure (Aminoff, 1961) with *N*-acetylneuraminic acid as a standard. Specific sugar components were identified by gas–liquid chromatography of alditol acetates (Sawardekar et al., 1965; Lehnhardt & Winzler, 1968) following mineral acid hydrolysis or by methanolysis of the sugars followed by conversion to the trimethyl silyl ethers (Clamp et al., 1972) prior to gas–liquid chromatography; the latter procedure only was employed for sialic acid. Glucosamine and galactosamine were also determined on the amino acid analyzer after acid hydrolysis in vacuo at 100 °C for 8 h in 4 N HCl; galactosaminitol was determined on the amino acid analyzer after acid hydrolysis as for the hexosamine analyses (Cheng & Boat, 1978). Asialoglycoprotein and asialoglycopeptides were prepared by incubation of the appropriate material at 37 °C with *Vibrio cholerae* sialidase (protease free) (Calbiochem). The incubation was carried out in 0.1 M sodium acetate, pH 5.6, containing 1 mM $CaCl_2$ and the material then was chromatographed on Sepharose 4B by utilizing 0.1 M acetate buffer, pH 5.0. The fractions containing sialic acid were combined and the identity of the released product as *N*-acetylneuraminic acid confirmed by comparison with authentic standards employing descending paper chromatography on Whatman no. 1 paper with *N*-butyl acetate:glacial acetic acid:H_2O (3:2:1) as solvent. In general, exclusion columns were calibrated with blue dextran (V) or [^{14}C]glucose (S).

Protein was determined by the procedure of Lowry (Lowry et al., 1951) or when screening chromatographic column effluents by a method employing Coomassie Brilliant Blue G (Sigma Chemical Co.) (Bradford, 1976). Ninhydrin was used to assay for free amino groups following Pronase digestion. Amino acid analyses were performed by ion-exchange chromatography with a Beckman Model 120C or 121 amino acid analyzer following hydrolysis of the samples in vacuo for 24 h at 110 °C with 6 N HCl.

Reduction and S-Carboxymethylation. Reduction and S-carboxymethylation of the glycoprotein were performed according to a modification of the method of Crestfield (Crestfield et al., 1963). The glycoprotein (2 mg) was dissolved in 1.4 M Tris-HCl, pH 8.6 (10 mL), containing 10 mM EDTA, 5.7 M guanidinium chloride, and 0.2 M DTT. The samples were incubated at 25 °C for 4 h and the reduced glycoprotein was reacted with iodoacetic acid (0.25 g) and 2 M NaOH (200 μL) for 45 min at room temperature in the dark. The reaction mixtures were then extensively dialyzed at 4 °C in the dark against H_2O.

Pronase Digestion. Digestion of 5 mg of glycoprotein in 1.0 mL of 0.1 M borate buffer, pH 8.0, containing 0.01 M calcium acetate was carried out at 37 °C for 72 h with the addition of 0.5 mg of Pronase (Calbiochem)/10 mg of glycoprotein at times zero, 24, and 48 h. A trace amount of toluene was added to prevent microbial contamination. The Pronase digestion was also carried out in 50 mM Tris buffer, pH 8.0, 0.01 M calcium acetate and in an independent digestion, Proteinase K (EM Biochemicals) (Ebeling et al., 1974) was used in place of Pronase. Exclusion chromatography of the protease digest was initially performed at either pH 5.2 in 0.1 M pyridine acetate with Sephadex G-25 (Pharmacia Fine Chemicals) or at pH 5.0 in 0.1 M acetate buffer by utilizing Sepharose 4B. The void volume fraction of the G-25 column was assayed for blood group activity (Morgan & Watkins, 1951). Anion-exchange chromatography was performed by using a pyridine acetate gradient on DEAE-cellulose (Whatman Biochemicals, Ltd.).

Electrophoretic Studies. Polyacrylamide gel electrophoresis was performed on 6% polyacrylamide gels in the presence of sodium dodecyl sulfate ± β-mercaptoethanol at 7 V/cm of gel (Weber et al., 1972). Samples (15–20 μg) were incubated in $NaDodSO_4$ ± BME for 2 h at 40 °C and then applied to the gels. Electrophoresis was also performed with gels containing 0.5% agarose (Bio-Rad Laboratories) and 1.5% acrylamide (Bio-Rad Laboratories) at 2.5 mA/tube for 2 h (Holden et al., 1971). Samples of 200–500 μg were applied to the agarose–acrylamide gels. Gels were stained for protein with Coomassie Brilliant Blue R (Sigma Chemical Co.) and for carbohydrate by the periodate–Schiff method (Zacharius et al., 1969).

Sedimentation Equilibrium Studies. The glycoprotein and the pronase resistant glycopeptide core were examined by analytical equilibrium ultracentrifugation employing the meniscus depletion method (Yphantis, 1964). Details of the conditions used are given in the appropriate figure legend. Prior to centrifugation, the sample in buffer was dialyzed for 24 h against the same buffer. The partial specific volume was not explicitly measured for this glycoprotein. Based on the amino acid analysis and carbohydrate composition, a figure of 0.60 was estimated; the volume for the glycopeptide was estimated to be 0.54.

Circular Dichroism. Spectra were recorded at 23 °C from 300 to 205 nm with a Cary Model 60 recording spectropolarimeter equipped with a CD attachment. The glycoprotein or glycopeptide was dissolved at a concentration of 1 mg/mL in 0.1 M NaCl or H_2O, respectively. The spectrum of the solvent was recorded before and after each experiment to correct for any base-line drift.

Alkaline Borohydride Treatment. A solution of 4 mg of glycoprotein in 2 mL of 0.05 M KOH–1.0 M $NaBH_4$ was incubated for 72 h at 37 °C (Carlson, 1966). The excess borohydride was eliminated by adjusting the pH to 4.5 at 4 °C by addition of 4 M acetic acid. The solution was evaporated three times with methanol and the resulting material examined by chromatography on a P10 column (Bio-Rad Laboratories) in 0.1 M pyridine–acetate buffer, pH 5.2. In some experiments, NaB^3H_4 (NEN) was employed to permit labeling of the oligosaccharide chains.

endo-α-Acetylgalactosaminidase Digestion of the Glycoprotein. Treatment of 2.0 mg of the desialylated glycoprotein

[1] Abbreviations used: $NaDodSO_4$, sodium dodecyl sulfate; BME, β-mercaptoethanol; PAS, periodic acid Schiff; DDT, dithiotreitol.

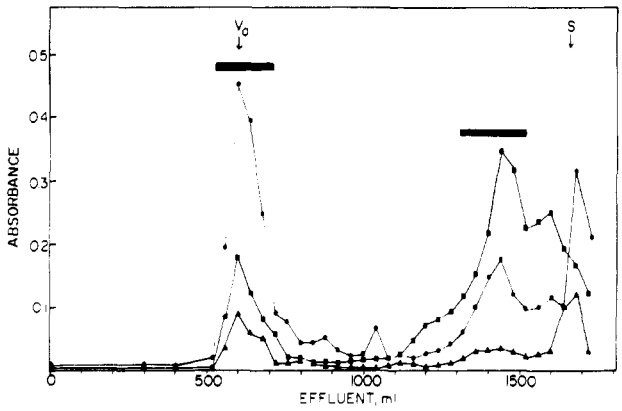


FIGURE 1: Sepharose 4B chromatography of the supernatant from the diluted human bronchial secretion. The column (5 × 92 cm) was operated with a pressure head of 50 cm at a flow rate of 100 mL/h. The eluting buffer was 0.1 M sodium acetate, pH 5.0. Representative aliquots were analyzed for neutral sugar (●), protein (■), and sialic acid (▲). Absorbance data are at 490, 660, and 550 nm, respectively. The bars show the fractions that were combined for further study.

Table I: Amino Acid Analysis of Bronchial Secretion after Chromatography on Sepharose 4B

amino acid	residues thousand fraction I	residues fraction II	glycopeptide (Pronase resistant core)
Lys	29	64	17
His	21	22	26
Arg	40	40	28
Asp	70	115	35
Thr	170	59	302
Ser	110	59	186
Glu	78	138	44
Pro	103	79	105
Gly	72	63	72
Ala	86	75	92
$^1/_2$-cystine	15	35	trace
Val	69	66	33
Met	10	11	
Ile	29	22	9
Leu	63	85	36
Tyr	11	27	6
Phe	21	41	9
glucosamine[a]	14.4%	5.1%	19.1%
galactosamine[a]	13.3%	1.0%	17.7%

[a] Data are expressed as percent by weight of the total glycoprotein.

with an *endo*-α-*N*-acetylgalactosaminidase isolated from *Diplococcus pneumoniae* (Bhavanandan et al., 1976) was carried out at 37 °C for 24 h in 1 mL of 0.1 M Tris–maleate buffer, pH 7.6. After the incubation, aliquots of the mixture were assayed for Morgan–Elson positive material by using authentic Galβ1→3GalNAc as standard (Reissig et al., 1955). The released oligosaccharide product was further identified by paper chromatography on Whatman no. 1 paper by using *N*-butyl acetate:glacial acetic acid:H_2O (3:2:1) as a solvent.

End-Group Analysis. Amino-terminal end-group analysis was performed by utilizing dansyl chloride (Gray, 1967). Following hydrolysis with 6 N HCl for 18 h, the products were examined by two-dimensional thin-layer chromatography on polyamide sheets (Woods & Wang, 1967).

Results

Purification and Physical Properties of the Mucin-Type Glycoprotein. After the low-speed centrifugation (7000*g*) of the bronchial secretion, 93% of the neutral sugar and 88% of the sialic acid (thiobarbituric acid analysis) remained in the supernatant. This supernatant was analyzed by exclusion chromatography on a Sepharose 4B column. The mucin-type glycoprotein emerged in the void volume (Figure 1) which also contained nearly all of the blood group A activity. The amino acid analysis of the void volume (500–700 mL) is presented in Table I; the total amino sugar content comprised 28% by weight of the glycoprotein; sulfate content was 4.5%. Fraction II (1300–1500 mL), when analyzed for amino acids, gave a profile more characteristic of serum-type glycoproteins (Table I). In addition, the presence of mannose, alkali stability of the peptide–oligosaccharide linkage, and extensive reactivity with anti-human serum following immunoelectrophoresis identified the components present as primarily circulating glycoproteins. The possibility exists that lung cell specified macromolecules are also to be found in this mixture. However, this fraction was not further studied.

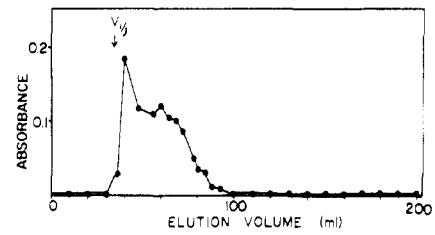


FIGURE 2: Bio-Gel A-150m chromatography of human bronchial mucin (Sepharose 4B-pk I, Figure 1). The column (1.5 × 55 cm) was operated in 0.1 M sodium acetate buffer, pH 5.0, at a pressure head of 30 cm and a flow rate of 9 mL/h. The column fractions were assayed for neutral sugar; absorbance data are at 490 nm.

Sepharose 4B gel filtration of the mucin-type glycoprotein preincubated with DTT and run in its presence showed no change in the elution pattern. This result suggests that reduction of disulfide bridges does not lead to a large decrease in molecular size. The peak I material from the Sepharose 4B column was subsequently chromatographed on a Bio-Gel A-150 M (Bio-Rad Laboratories) column (1.5 × 55 cm) by utilizing pH 5.0, 0.1 M acetate buffer. The elution pattern showed some retention of the glycoprotein (Figure 2), but substantial material was still present in the void volume. The heterogeneous profile seen in the Bio-Gel A-150 M column elution pattern may represent aggregation, entrapment, peptide and/or saccharide polydispersity, or some combination of these.

End-group analysis of fraction I revealed no measurable amino-terminal residue. This result is compatible with the properties of some glycoproteins where the N-terminal amino acid is found to be blocked, e.g., α_1-acid glycoprotein (Schmid, 1954). Additionally, based on the amount of material employed and the detection sensitivity of the analytical technique, the results indicate the absence of significant contamination by proteins with accessible termini.

The molecular weight of fraction I was examined by equilibrium sedimentation ultracentrifugation at three different concentrations in 0.5 M NaCl (Figure 3). Employing a partial specific volume of 0.60, a minimum molecular weight of 1.8×10^6 can be estimated, although variation in the frequency of saccharide chains as well as their nature introduces an uncertain level of heterogeneity. The upward displacement of the plot of σ_W vs. concentration is indicative of the presence of species of higher molecular weight. An evaluation of the data by means of a two species plot (Roark & Yphantis, 1969) gave no indication of specific association stoichiometry, although components with M_W of 10^7 or higher are apparently present. These are likely to be aggregates but may arise in

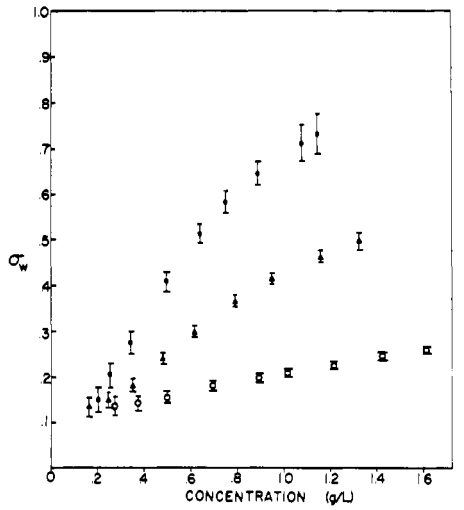


FIGURE 3: Equilibrium sedimentation analyses of the void volume peak described in Figure 1. The sample was run at 20 °C, 2000 rpm, and at three different loading concentrations: 0.25 (●), 0.5 (▲), and 1.0 mg/mL (O). Data were obtained 8 h after equilibrium was reached, 36 h after the initiation of the run. The molecular weight parameters were calculated by a computer program from fringe displacement patterns obtained after equilibrium was reached.

part from the heterogeneity present in the minimal species.

The mucin-type glycoprotein fraction recovered from the Sepharose 4B chromatography was completely excluded from 6% polyacrylamide gels when analyzed by $NaDodSO_4$–polyacrylamide gel electrophoresis (± β-mercaptoethanol). These results combined with those from the ultracentrifugation study indicated a high "native" molecular weight. The limitations of this type of molecular weight estimation, however, preclude any statements regarding the role of disulfide bonds in the macromolecule. Accordingly, the glycoprotein was reduced with dithiothreitol in the presence of guanidinium chloride and any -SH groups generated by reduction were carboxymethylated with iodoacetamide. The bulk of the material, approximately 80%, still appeared at the void volume of a Sepharose 4B column, although a minor component may be slightly included. The native and reduced–carboxymethylated glycoproteins were further examined by electrophoresis in 0.5% agarose–1.5% polyacrylamide gels. The native glycoprotein did not enter this gel and no lower molecular weight protein or carbohydrate components were detected after loading the gels with 500 μg of glycoprotein. The reduced glycoprotein showed two components corresponding in proportion to those observed on Sepharose 4B chromatography. The included component stained weakly, but detectably, with the periodic acid–Schiff reagent and is presumably a glycoprotein.

Chemical Composition. The carbohydrate analysis of the material in the void volume of the 4B column is presented in Table II. The overall carbohydrate content was estimated to represent 70% by weight of the glycoprotein. The sialic acid was identified by paper chromatography as *N*-acetylneuraminic acid and was completely susceptible to *Vibrio cholerae* neuraminidase. The fact that mannose was absent indicated that the material was free of glycoproteins wherein the carbohydrate moiety is attached via the amide nitrogen of asparagine.

The linkage of the oligosaccharides to the peptide core was concluded to be O-glycosidic based on the results of treatment with alkaline borohydride. The data indicate that two-thirds of both the serine and threonine residues are substituted. Chromatography of the reaction products on a P-10 column

Table II: Carbohydrate Composition of the Glycoprotein before and after Alkaline Borohydride Treatment

carbohydrate residue	molar ratio[a] glycoprotein	molar ratio[a] $OH-NaBH_4$ treated
L-fucose	2.6	2.9
D-galactose	3.1	2.7
N-acetyl-D-galactosamine	1.4	0.2
N-acetyl-D-glucosamine	2.0	1.8
sialic acid	1.0	1.0
N-acetyl-D-galactosaminitol	0.0	1.0

[a] Relative to sialic acid.

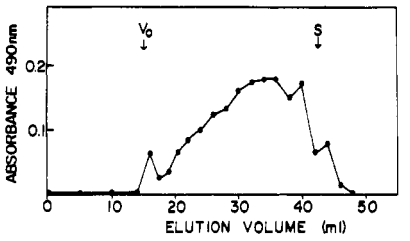


FIGURE 4: Bio-Gel P-10 chromatography of bronchial secretion (Sepharose 4B-pk I, Figure 1) which has been treated with $NaBH_4$–KOH. The column (1 × 50 cm) was run in 0.1 M pyridine–acetate buffer at a flow rate of 8 mL/h. Fractions were assayed for neutral sugar.

showed that essentially all of the neutral sugar was included (Figure 4); the peptide product of this reaction was not studied. Based on the chromatographic mobility of known oligosaccharides, the size of the eliminated products ranged from 2 to about 9 monosaccharides with the majority containing 5 to 7 sugars. About 85% of the *N*-acetylgalactosamine was reduced to galactosaminitol, indicating that GalNAc is the linkage sugar (Table II). The stoichiometry of the monosaccharides, the blood group A reactivity of the starting glycoprotein, and the heterogeneity shown on P-10 chromatography clearly indicate that several types of oligosaccharides are present.

Purification and Physical and Chemical Characterization of the Glycopeptides. Preliminary fractionation of the Pronase digest was performed on a Sephadex G-25 column. Over 90% of the material which was positive for neutral sugar eluted at the void volume and accounted for virtually all (96%) of the starting carbohydrate content. The column fractions contained no detectable protein based on the colorimetric procedure, although amino acids and small peptides were readily detected in a second peak eluting near the included volume. The glycopeptide material was excluded from both G-50 and controlled pore glass (10–240) columns. However, it was included on a Sepharose 4B column in sharp contrast to the starting material (Figure 5). The void volume fraction of the G-25 column was found to exhibit blood group A activity. This result was expected since the A determinants are in the carbohydrate structure. Quantitation of the A activity was not attempted on this fraction.

A second Pronase digestion performed in Tris buffer or digestion with proteinase K yielded material which failed to enter the G-25 gels but was included on Sepharose 4B. Incubation of the Pronase digest with dithiothreitol and chromatography on Sepharose 4B in the presence of dithiothreitol did not change the profile of carbohydrate reactive material.

The amino acid composition of the glycopeptide (Table I) shows a significant enrichment in threonine and serine (nearly 50% of the total), indicating that the large molecular weight of this fragment is due to clusters of serine and threonine

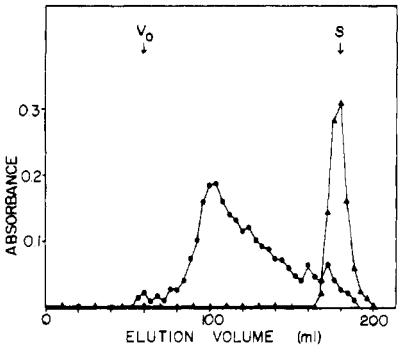


FIGURE 5: Sepharose 4B chromatography of a 72-h Pronase digest of human bronchial secretion (Sepharose 4B-pk I, Figure 1). The column (2 × 66 cm) was operated at a pressure head of 50 cm at a flow rate of 15 mL/h with 0.1 M sodium acetate buffer, pH 5.0. Fractions were analyzed for neutral sugar (●) and free amino groups (▲). Absorbance data are at 490 and 570 nm, respectively.

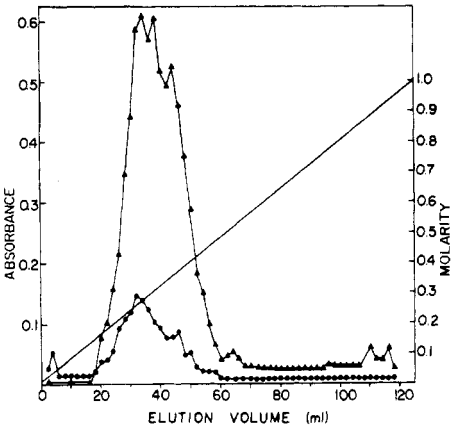


FIGURE 6: Chromatography on DEAE-cellulose of the pooled void volume fractions from G-25 chromatography of the Pronase digested material (Figure 5). The material was eluted with a linear gradient from 10 mM to 1 M pyridine–acetate, pH 5.2. Fractions were analyzed for neutral sugar (●) and sialic acid (▲). Absorbance data are at 490 and 550 nm, respectively.

residues protected from further proteolytic digestion by carbohydrate side chains.

The Pronase digestion material from the void volume of the G-25 was pooled, concentrated, and run on a DEAE-cellulose column. The material was eluted with a linear gradient from 10 mM to 1 M pyridine–acetate, pH 5.2. As illustrated in Figure 6, all of the material was eluted between 0.2 and 0.5 M pyridine–acetate but no clear fractionation was obtained. This result suggests that a distinction between sulfated and nonsulfated components may not be present.

$NaDodSO_4$–polyacrylamide gel electrophoresis (10 cm; 6% polyacrylamide gels ± β-mercaptoethanol) was carried out with the Pronase digested material (void volume of the G-25). There was no detectable protein (Coomassie Blue stain) but two bands contained carbohydrate and reacted with the PAS reagent. One of the bands was comprised of material which did not enter the gel, and the second band migrated about 1 cm. The results were identical whether or not β-mercaptoethanol was present.

Since the high carbohydrate content of glycopeptides makes molecular weight estimation unreliable by gel electrophoretic methods, the glycopeptide obtained after Pronase digestion was examined by ultracentrifugation. Fractions from the major peak of the Sepharose 4B chromatography of the unreduced glycopeptides were combined, dialyzed, lyophilized, resuspended in 50 mM Tris, pH 8.0, or 50 mM Tris, pH 8.0,

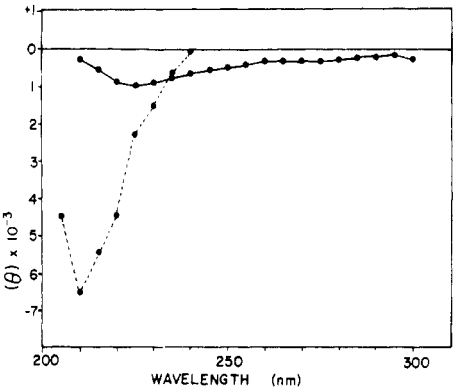


FIGURE 7: Circular dichroic spectrum of intact bronchial secretion (Sepharose 4B-pk I, Figure 1) (—) compared with the spectrum of the glycopeptide (---) core after Pronase digestion. Concentration was 1 mg/mL.

+ 4 M guanidinium chloride, and dialyzed against the appropriate buffer. This material was examined in the analytical ultracentrifuge under equilibrium conditions. Based on a partial specific volume of 0.54, the estimated molecular weight for the glycopeptides was 1.5×10^5; there was negligible polydispersity.

In an attempt to eliminate any aggregation of the glycopeptides which might be due to terminal sialyl residues, the Pronase digest was treated with sialidase and rechromatographed on Sepharose 4B. All detectable sialic acid was removed from the glycopeptides, but the elution profile was essentially unchanged. An ultracentrifugation study of the major glycopeptide peak following sialidase digestion gave an estimated molecular weight of 1.4×10^5, a value only slightly less than that for the starting glycopeptides. A further centrifugation study of the desialylated glycopeptide in the presence of 4 M guanidinium chloride gave similar results.

The conformation of the glycoprotein and the Pronase core were examined by circular dichroic measurements. The spectrum (Figure 7) for the intact glycoprotein shows a negative absorption trough at 225 nm; the glycopeptide obtained after Pronase digestion showed a shift in the absorption trough to 210 nm.

Discussion

Chemical characterization of the bronchial glycoproteins present in gel-like secretions requires a solubilization step prior to analysis. In order to prepare material as nearly native as possible, a mild procedure of isolation was used and neither detergent nor reducing agent was employed. Since nearly all of the carbohydrate was recovered in the soluble fraction, it may be concluded that the analytical data obtained are representative. Following solubilization, chromatography on Sepharose 4B separated the mucin type glycoprotein from lower molecular weight protein components. This was substantiated by the absence of lower molecular weight proteins upon $NaDodSO_4$ gel electrophoresis (±BME) of the mucin in 6% polyacrylamide gels. These data combined with the agarose–acrylamide electrophoresis results support the minimum molecular weight of 1.8×10^6 calculated for the mucin glycoprotein from the sedimentation equilibrium study.

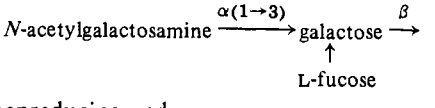
Hill et al. have proposed aggregation of subunits to account for the high molecular weight of native and asialo ovine submaxillary mucin. They attribute the aggregation to noncovalent interactions dependent upon the carbohydrate moieties of the mucin (Hill et al., 1977). The presence of disulfide bonds in bronchial mucin has also been suggested

by several investigators (Boat et al., 1976; Roberts, 1976; Creeth et al., 1977). The agarose–acrylamide electrophoresis indicates that a small portion (approximately 20%) of the native material is associated via disulfide bonds. However, the minimal size of the reduced product is estimated at 1×10^6, based on gel electrophoresis studies with submaxillary mucins (Holden et al., 1971).

An amino acid analysis of the mucin showed that serine and threonine comprise about one-third of the residues and together serine, threonine, proline, glycine, and alanine constitute more than 50% of the total amino acid content. These analytical data are somewhat lower than those reported for other mucins or from bronchial glycoproteins obtained from a patient with cystic fibrosis (Roussel et al., 1975). In the latter case, the amino acid composition was comparable to that of the glycopeptide obtained after Pronase digestion in this study. The carbohydrates galactose, fucose, glucosamine, galactosamine, and sialic acid are present in a molar ratio of 3:3:2:2:1. Sulfate is presumably esterified to the galactose moieties (Havez et al., 1968; Roussel et al., 1972, 1975; Boat et al., 1976) but detailed localization of the sulfate ester groups was not achieved in this study. Mannose and uronic acid which are constituents of serum glycoproteins and proteoglycans, respectively, were not present. The mucin was found to exhibit blood group A activity (Watkins, 1972). A number of additional bronchial mucus samples have been obtained from pediatric patients hospitalized for surgery or other reasons not involving the pulmonary system. These have all reflected the blood group status of the donor and presumably are characteristic epithelial cell products of secretors.

The carbohydrate–protein linkages were shown by β elimination to involve *N*-acetyl-D-galactosamine attached to serine and threonine via *O*-glycosyl bonds. Approximately two-thirds of the serine and threonine residues were substituted; thus approximately one of every five amino acids has a saccharide chain attached. The structures of the carbohydrate prosthetic groups have not been fully defined but the results of the P-10 chromatography indicate a heterogeneous size distribution (2–9 sugars). A small portion (7%) of the oligosaccharides are present as galactosyl-β1→3-*N*-acetylgalactosaminyl disaccharides linked to the protein core through serine and threonine residues. This conclusion is based on the results of digestion of the glycoprotein with an *endo*-α-*N*-acetylgalactosaminidase from *Diplococcus pneumoniae* (Umemoto et al., 1977). Since the enzyme preparation employed was free of sialidase, *exo*-acetylhexosaminidase, galactosidase, and fucosidase activities and acts only on unsubstituted disaccharidyl substrates, it may be inferred that this structural entity arises from incomplete chains. The Gal→GalNAc disaccharide is commonly present in mucin type glycoproteins.

Due to the blood group A activity, some of the oligosaccharides should have the sequence



at their nonreducing end.

The results of the Pronase digestion indicate that the entire carbohydrate content is localized in a serine–threonine rich region of the polypeptide. It may be estimated that the polypeptide moiety of the glycopeptide obtained after Pronase digestion contains about 100 amino acids of which about half are substituted with saccharide chains. Reconciling these data with the molecular weight estimates of the native mucin requires either noncovalent associative behavior of the saccharide-free domains or the presence of several sections of high sugar content and spacer regions completely free of carbohydrate; composite structures are also feasible. Glycoprotein structural models composed of two types of regions have been proposed for blood group substances (Dunstone & Morgan, 1965; Kristiansen & Porath, 1968; Donald, 1973; Goodwin & Watkins, 1974) and for the epithelial secretions of bovine (Bhushana Rao & Masson, 1977) and human (Roberts, 1976) origin. Additionally, the clustering of serine and threonine amino acid residues has been observed in the regions of the blood group substances carrying the carbohydrate chains (Goodwin & Watkins, 1974).

The high molecular weight of the glycopeptide fraction is not due to aggregation caused by the presence of the negatively charged terminal sialic acid residues, to ionic interactions or to hydrogen bonding.

A distinctive characteristic of the circular dichroic spectra of both the intact mucin and the glycopeptide fraction was a negative trough at 210 mM for the glycopeptide fraction, similar to the results reported for milk oligosaccharides (Kabat et al., 1969). The intensity of the negative absorption observed after Pronase digestion of the mucin indicates that the spectrum is largely dependent on the carbohydrate moiety of the glycopeptide.

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