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Purification, Properties, and Partial Structure Elucidation of a High-Molecular-Weight Glycoprotein from Cervical Mucus of the Bonnet Monkey (*Macaca radiata*)†

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ABSTRACT: A high-molecular-weight glycoprotein has been purified from the cervical mucus of the bonnet monkey (*Macaca radiata*). The glycoprotein was shown to be homogeneous by electrophoresis, sedimentation equilibrium, and N-terminal group determination, and to contain 19% protein, 19% D-galactose, 18% *N*-acetyl-D-galactosamine, 15% *N*-acetyl-D-glucosamine, 11% L-fucose, 10% sialic acid, and 1% sulfate groups, corresponding to about 1800 amino acid residues and 400 carbohydrate side chains of about 9 monosaccharides. The carbohydrate chains are linked to the peptide backbone through *N*-acetyl-D-galactosamine and serine (or threonine) residues. Reduction with dithiothreitol and alkylation with iodoacetic acid reduced the molecular mass from 1 to 0.5×10^6 daltons and produced subunits having the same size, charge, and N-terminal amino acid. Electrophoretic studies suggested the presence of disulfide bonds between two chains of the glycoprotein. Degradation with alkaline borohydride gave, after fractionation on Bio-Gel P-2, fractions containing L-fucose, D-galactose, *N*-acetyl-D-galactosaminitol, *N*-acetyl-D-galactosamine, *N*-acetyl-D-glucosamine, and sialic acid in the ratio of 1.0:3.0:1.0:1.0:1.3:1.0. Further fractionation by electrophoresis and paper chromatography gave a charged fraction representing 13% of the original glycoprotein. Enzymic degradation and methylation studies indicated the presence of the structure α-Gal-(1→3)-[Fuc(1→2)]-Gal-(1→4)-GlcNAc, linked to a core component containing *N*-acetyl-D-galactosaminitol.

Cervical mucus is a hydrophilic, gel-like substance which contains soluble proteins and inorganic ions and is classified as "epithelial mucin" (Pigman et al., 1974; Pigman and Moschera, 1973; Montgomery, 1970; Spiro, 1970). From crude mucus collected from pregnant animals at estrus, Gibbons (1959) has isolated a bovine cervical mucoid consisting of 75–80% carbohydrates and 20–25% amino acid residues; the sialic acid residues were shown to be one of the factors controlling the physical properties of the mucin, and various carbohydrate contents of the cervical secretion have been ob-

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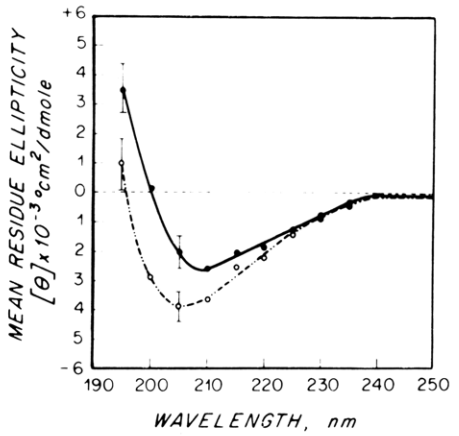

FIGURE 1: Circular dichroic spectrum of intact glycoprotein (—) compared with the reduced and *S*-carboxymethylated glycoprotein (- - -).

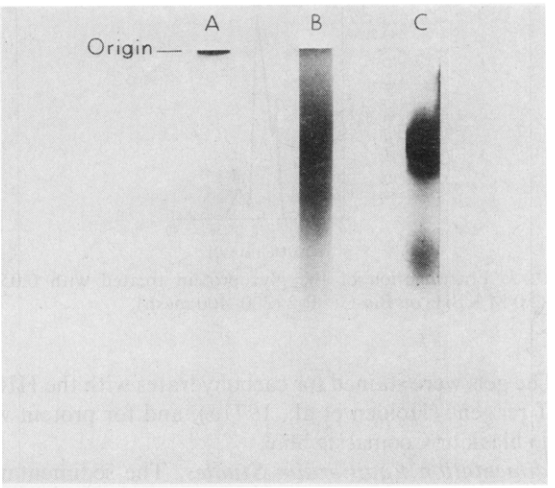

FIGURE 2: Agarose–polyacrylamide (0.5–1.5%) electropherograms of the glycoprotein (200–300 μg) stained for protein with Amido black (A) and for carbohydrate with PAS (B); and of the partially reduced glycoprotein (200–300 μg) stained with PAS (C). The samples were run toward the anode (bottom of picture).

served at different stages of ovarian function (Gibbons, 1959; Iacobelli et al., 1971). One of the roles of human cervical mucus is to be receptive to spermatozoa at or near the time of ovulation and to impede their entry at other times. The spermatozoa are both protected and possibly capacitated by the mucus, which supplements their energy requirements (Moghissi and Blandau, 1972).

In order to understand the role that cervical mucus plays in the treatment of spermatozoa in primates, we undertook the purification, characterization, and partial structure identification of the major macromolecular component of the cervical mucus of the bonnet monkey (*Macaca radiata*). This animal was selected because it secretes large amounts of mucus, and its reproductive cycle is similar to the human cycle.

Experimental Section

Materials. The mucus was aspirated from the cervix and vagina of the bonnet monkey with the aid of a suction pump. The crude mucus was stored in preweighed vials at −20 °C.

Analytical Methods. D-Galactose, L-fucose, *N*-acetyl-D-galactosamine, *N*-acetyl-D-glucosamine, and *N*-acetyl-D-galactosaminitol were determined by gas–liquid chromatography on lyophilized samples (Reinhold, 1972). The bound sialic acid was released with 25 mM H_2SO_4 for 60 min at 80 °C and measured by the method of Warren (1959).

N-Acetylneuraminic acid and *N*-glycolyneuraminic acid were determined by hydrolysis of the glycoprotein (20 mg) with *Clostridium perfringens* neuraminidase (Worthington) (2 mg) in 0.1 M sodium acetate buffer, pH 5.0, at 37 °C for 48 h and dialysis. The dialysate was desalted by passage through a column of Dowex 50W-X8 (2 × 20 cm) cation-exchange resin and lyophilized. The residue as well as samples of standard *N*-acetylneuraminic acid and *N*-glycolylneuraminic acid were applied to Whatman paper No. 3 and irrigated for 17 h with butanol–propanol–0.1 M HCl (1:2:1, v/v). The part of the paper containing the standard of *N*-acetylneuraminic acid and *N*-glycolyneuraminic acid was treated with the HIO_4–benzidine reagent (Smith, 1960). The samples investigated were eluted with H_2O, and free *N*-glycolyneuraminic acid and *N*-acetylneuraminic acid were determined by the method of Warren (1959) and by gas–liquid chromatography after treatment with 0.5 M methanol–HCl for 1 h and per-(*O*-trimethylsilyl)ation with Trisyl (Supelco) for 60 min at room temperature. The nondialyzable material was lyophilized and the bound sialic acid determined. Protein determinations were measured by the method of Lowry et al. (1951).

Sulfate Determination. The inorganic sulfate ions were determined with the ultramicro method of Spencer (1960).

Amino Acid Analysis. The amino acid composition of the purified cervical glycoprotein was determined with a Beckman Model 120B amino acid analyzer after hydrolysis of the samples with 6 M HCl for 18 h at 110 °C in vacuo. In order to determine the conversion, by alkaline β elimination, of serine and threonine to alanine and α-aminobutyric acid, respectively, the glycoprotein (17 mg) was treated with 0.07 M KOH–2 M KBH_4 (7.0 mL) for 18 h at 45 °C. To an aliquot (3.5 mL) of the sample was added 0.08 $PdCl_2 \cdot 2H_2O$ (0.2 mL) (Tanaka and Pigman, 1965). The suspension was kept for 90 min at room temperature and the precipitated Pd removed by centrifugation. Acetic acid and methanol were added to the sample and the borate ions were removed in vacuo. The sample was evaporated to dryness in a N_2 atmosphere and hydrolyzed for 18 h at 110 °C with 6 M HCl.

Circular Dichroism. The spectra (Figure 1) were recorded at 23 °C with a Cary Model 60 recording spectropolarimeter equipped with a Cary Model 6001 CD attachment. The purified glycoprotein or *S*-carboxymethylated glycoprotein (0.25 mg) was dissolved in either H_2O or 0.1 M phosphate buffer at a pH varying from 4.3 to 9.0. The spectra of H_2O or of the appropriate phosphate buffers were recorded before and after each experiment to correct for any shift. The data are expressed in terms of $(\theta)_{MRW}$ based on a glycoprotein residue weight of 172, which was calculated from the amino acid composition and the proportions of *N*-acetyl-D-galactosamine, *N*-acetyl-D-glucosamine, and *N*-acetylneuraminic acid.

N-Terminal Amino Acid Analysis. The N-terminal amino acid residue was identified with the dansylation method of Gray and Hartley (1963) by Dr. P. Roussel (Unité des Protéines, INSERM, Lille, France).

Electrophoretic Studies. Polyacrylamide electrophoresis was performed on gels containing 0.5% agarose (General Biochemicals) and 1.5% acrylamide (Cyanogum 41, Fisher) (see Figure 2) or 7.5% acrylamide (Holden et al., 1971a). The glycoprotein was reduced with 0.4 M dithiothreitol at 23 °C for 2 h and samples weighing 200–300 μg and 1.0–1.2 mg were applied to agarose–1.5% acrylamide and to 7.5% acrylamide, respectively. The presence of a basic protein was examined on gels containing 7.5% acrylamide in 0.2 M citrate buffer, pH

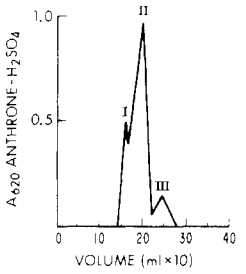

FIGURE 3: Fractionation of the glycoprotein treated with 0.05 M KOH-2.0 M KBH_4 on Bio-Gel P-2 (200-400 mesh).

4.0. The gels were stained for carbohydrates with the HIO_4-Schiff reagent (Holden et al., 1971a), and for protein with Amido black or Coomassie blue.

Sedimentation Equilibrium Studies. The sedimentation equilibrium was determined, on solutions of purified monkey cervical glycoprotein (2 mg), in 1 mL of 6 M guanidine hydrochloride (Heico)-0.05 M Tris[1]-HCl, pH 7.0, and 0.1 mM dithiothreitol, dialyzed for 48 h against the same buffer, by Dr. D. O'Hara (Massachusetts General Hospital, Boston) with the meniscus-depletion sedimentation method of Yphantis (1964) on a Model E ultracentrifuge. A value of 0.637 for the partial specific volume was calculated from the amino acid and carbohydrate composition and used in the determination. The data were analyzed with a computer program provided by Drs. Roark and Yphantis. The molecular weights were calculated by extrapolation of the point-average molecular weights to infinite dilution.

Purification of Monkey Cervical Glycoprotein. The crude mucus (3 g, wet material) was partially dissolved by being stirred overnight at room temperature in 0.05 M phosphate buffer at pH 7.0 (200 mL). The undissolved material was centrifuged off, and the clear supernatant was applied to a Bio-Gel P-200 (50-100 mesh) column (5 × 60 cm), previously equilibrated with the same buffer. The carbohydrate-containing material was pooled, dialyzed extensively against H_2O, and lyophilized.

Reduction and S-Carboxymethylation of Monkey Cervical Glycoprotein. The reduction and S-carboxymethylation were performed according to a modified method of Crestfield et al. (1963). The glycoprotein (2 mg) was dissolved in 1.4 M Tris-HCl buffer (1 mL), pH 8.6, containing EDTA (30 mg), guanidine hydrochloride (Heico) (5.5 g), and dithiothreitol (0.3 g) corresponding to a 0.1 M concentration. The control sample did not contain dithiothreitol. The samples were incubated at 25 °C for 4 h. The S-carboxymethylation was performed on aliquots (1 mL) from the sample, precaution being taken to exclude light during all steps. Iodoacetic acid and 2 M NaOH (20 μL) were added to the reduced sample. The molar concentration of iodoacetic acid was slightly less than that of dithiothreitol. The alkylation was performed at room temperature for 45 min. The samples were extensively dialyzed against H_2O in the dark for 24 h.

Oligosaccharides Liberated by Alkaline Borohydride Treatment. The glycoprotein (600 mg) was treated with 0.05 M KOH-2.0 M KBH_4 for 18 h at 45 °C in a final volume of 400 mL (Iyer and Carlson, 1971). The excess of borohydride was eliminated by addition of acetic acid at 4 °C. The sample was desalted with Ag 50-X8 (H^+, 200-400 mesh, Bio-Rad) cation-exchange resin, at 4 °C, and the aqueous solution was

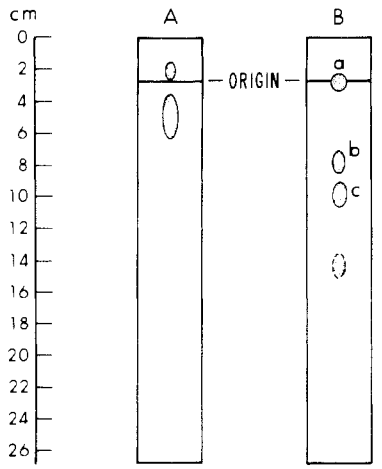

FIGURE 4: (A) Separation by high-voltage electrophoresis of fraction II obtained by gel filtration of reduced glycoprotein on Bio-Gel P-2. (B) Paper chromatography on Whatman No. 3 MM paper of acidic fraction obtained by electrophoresis.

neutralized by addition of pyridine and evaporated. Alternatively, the sample was desalted on a column (5 × 70 cm) of Bio-Gel P-2 (200-400 mesh) and the salt-free solution was lyophilized.

The residue was separated into fractions I, II, and III by gel filtration on a column (2.5 × 110 cm) of Bio-Gel P-2 (200-400 mesh) equilibrated with 0.05 M pyridine-acetate, pH 5.0 (see Figure 3). Fractions of 1 mL were collected and aliquots from each tube were analyzed with the anthrone-H_2SO_4 reagent. The resulting three fractions were lyophilized.

The neutral and charged oligosaccharides of fraction II were separated by high-voltage electrophoresis on Whatman paper No. 3 MM in 0.05 M sodium acetate buffer, pH 7.5, at 3000 V for 1.5 h. Strips of paper were stained with the HIO_4-benzidine reagent (Smith, 1960) (Figure 4A). The remaining samples were eluted by capillary action with H_2O, desalted on a column (0.5 × 60 cm) of Bio-Gel P-2 (200-400 mesh), and lyophilized. The acidic fraction obtained by electrophoresis was further fractionated by paper chromatography on Whatman No. 3 MM paper in either butanol-pyridine-H_2O (5:1:4, v/v) or butanol-propanol-0.1 M HCl (1:2:1, v/v) for 240 h (Figure 4B). Strips of the paper were stained with the HIO_4-benzidine reagent (Smith, 1960), and the remaining samples were eluted by capillary action with H_2O. In order to examine the purity of the oligosaccharides, paper chromatography with butanol-pyridine-H_2O (5:1:4, v/v) and butanol-propanol-0.1 M HCl (1:2:1, v/v) and high-voltage electrophoresis were performed on solutions in 0.05 M $Na_2B_4O_7$ buffer, pH 9.3, for 60 min at 2000 V on Whatman No. 3 MM paper, and the spots were detected as just described (Smith, 1960).

Periodate Oxidation of Oligosaccharides. The sample (400 μg), dissolved in 0.1 M sodium acetate buffer (pH 5.8, 0.2 mL) containing 0.15 M NaCl and 0.09 M $NaIO_4$, was incubated for 18 h at 24 °C. The reaction was stopped by addition of an excess of 1,2-ethanediol, and the sample was desalted by chromatography on a column (0.5 × 60 cm) of Bio-Gel P-2 (200-400 mesh) equilibrated with 0.05 M acetic acid. The sugar composition was determined by gas-liquid chromatography before and after oxidation.

Degradation of Oligosaccharides with α-Galactosidase (Ficin). α-Galactosidase was purified from Ficin with the method of Hakomori et al. (1971). To each 500 μg of sample dissolved in 0.1 M sodium citrate buffer, pH 4.5, were added

[1] Abbreviations used: Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; PAS, periodic acid-Schiff.

2-4 units of α-galactosidase. One unit of enzyme activity was defined as the amount that hydrolyzes 1 mol of *p*-nitrophenyl α-D-galactopyranoside per h. The samples were incubated at 37 °C under toluene for 150 h, and the D-galactose liberated was measured with galactose dehydrogenase (Kornfeld et al., 1955).

Removal of L-Fucose and Sialic Acid from Oligosaccharides. L-Fucose was released by treatment with 0.5 M H_2SO_4 for 1 or 2 h at 80 °C and separated on a calibrated column (0.5 × 60 cm) of Bio-Gel P-2 equilibrated with 0.05 M acetic acid. The released and bound L-fucose was determined according to Dische and Shettles (1948). The D-galactose released was determined by the anthrone method (Dreywood, 1946). Sialic acid was released by treatment with 25 mM H_2SO_4 for 1 h at 80 °C and determined with the method of Warren (1959).

Methylation Studies. The oligosaccharide or glycopeptide sample (0.1-2.0 mg) was methylated with the method of Hakomori (1964). It was dissolved in dimethyl sulfoxide (1.0 mL), methylsulfinylmethylsodium (0.1 mg per mg of sample) was added, and the mixture was stirred at room temperature for 1 h in a N_2 atmosphere. Four stepwise additions of methyl iodide (each 10 μL/mg of sample) were performed in a 2-h period under N_2, and the solution was stored overnight under N_2. After addition of H_2O (3 mL), the aqueous solution was transferred onto a charcoal (Norit-A)-Celite 535 (2:1, w/w) column. In order to remove the dimethyl sulfoxide and the salts, the column was washed with H_2O, methanol-H_2O (1:1), and methanol. The methylated compound was eluted with 80 mL of chloroform-methanol (2:1, v/v). After removal of the solvent by evaporation, the samples were hydrolyzed with 3 M trifluoroacetic acid for 4 h at 105 °C under nitrogen. The trifluoroacetic acid was removed by evaporation in the presence of NaOH pellets in a vacuum desiccator. The reduction of the aldehyde group of the free, methylated sugars was performed overnight at room temperature with 1 M $NaBD_4$ (0.5 mL). The excess of $NaBD_4$ was destroyed by addition of acetic acid to pH 5.0. The reduced samples were applied to a column (1.2 × 1.8 cm) of Dowex 50W-X8 (H^+, 100-200 mesh) cation-exchange resin, previously washed with H_2O. The alditols were eluted with water and the hexosaminitols with 1 M NH_4OH. Each fraction was dried under reduced pressure, and the alditols were acetylated with excess acetic anhydride-pyridine (1:1, v/v) for 1 h at room temperature under N_2. The free hexosaminitols were acetylated with excess acetic anhydride-pyridine (1:1, v/v) for 3 h at 100 °C under N_2. In both cases, the excess of acetic anhydride-pyridine was removed by evaporation in a desiccator over NaOH pellets and H_2SO_4. The alditol acetates of the methylated sugars were dissolved in chloroform and analyzed by gas-liquid chromatography on a stainless-steel column (⅓ m × 1.5 m) of 0.05% ECNSS-M on GLC 110 (120-140 mesh, Supelco) at a flow rate of ca. 40 mL/min with a temperature raising 10 °C per min from 110 °C. The hexosaminitol acetates were analyzed isothermally at 210 °C. The Perkin-Elmer 900 gas chromatograph was connected by means of a fritted-glass helium separator to a Hitachi-Perkin-Elmer mass spectrometer RMU-6L on line with an IBM 1800 computer. The mass spectra were recorded with the separator temperature at 250 °C, the ionization potential at 70 EV, and the temperature of the ion source at 250 °C.

Results

Purification, Physical Properties, and N-Terminal Amino Acid of the Cervical Glycoprotein. The crude cervical mucus was separated by gel filtration on Bio-Gel P-200 into a single, anthrone-positive component with a recovery of 75-85% of the starting material. The figure describing the separation was submitted to the scrutiny of the reviewers and will be furnished by writing directly to the authors. The loss of material was attributed to material being adsorbed to the Bio-Gel column.

TABLE I: Composition of Monkey Cervical Glycoprotein.

Component	%	Molar Ratio[a]
L-Fucose	11.0	1.0
D-Galactose	19.0	1.5
N-Acetyl-D-galactosamine	18.0	1.2
N-Acetyl-D-glucosamine	15.0	1.0
Sialic acid	10.0	1.4
Protein	19.0	
Sulfate	1.0	0.2

[a] Relative to *N*-acetyl-D-glucosamine.

The purity of the glycoprotein purified on Bio-Gel P-200 was studied by electrophoresis on 0.5% agarose-1.5% polyacrylamide. No bands were observed after staining for protein with Amido black (Figure 2A); Holden et al. (1971a) had demonstrated that under similar conditions purified bovine, ovine, or porcine submaxillary mucins do not stain with Amido black, but, when the electrophoresis was performed with unpurified mucins, contaminating proteins were detected with the same reagent. Similarly, electrophoresis of the reduced glycoprotein (even after overloading the gels with 1.2 mg of glycoprotein) showed no detectable, contaminating proteins with Amido black. Staining for carbohydrate with the HIO_4-Schiff's reagent revealed a single, diffuse band for the purified glycoprotein (Figure 2B), whereas staining of the reduced glycoprotein showed two components (Figure 2C). This formation of two compounds may be explained by the presence of stabilized disulfide bonds; the faster moving electrophoretic component would be a small, dithiothreitol-reduced subunit of the glycoprotein, whereas the slower moving component would be the unreduced portion of the glycoprotein. This hypothesis was confirmed by the sedimentation equilibrium study.

The purity of the glycoprotein was further evaluated by the determination of the sedimentation equilibrium. No heterogeneity was observed for the glycoprotein purified on Bio-Gel P-200; a plot of log c vs. $r^2/2$ for the entire cell had a correlation coefficient greater than 0.999. Molecular mass values of 9.9×10^5 and of 1.03×10^6 daltons were calculated for the intact glycoprotein respectively; the values were derived from the computer analysis of the data. A molecular weight of 4.6×10^5 was calculated for the glycoprotein reduced with 0.1 M dithiothreitol. Analysis of the N-terminal amino acid residue of the intact and 0.1 M dithiothreitol-reduced glycoprotein indicated that both samples possess only an arginine residue as N-terminal group. Thus, no contaminating proteins were found in the glycoprotein purified by gel filtration on Bio-Gel P-200 by agarose-polyacrylamide electrophoresis, sedimentation equilibrium study, and N-terminal amino acid analysis.

Chemical Composition. The glycoprotein contained 73% of carbohydrate which includes D-galactose, L-fucose, *N*-acetyl-D-galactosamine, *N*-acetyl-D-glucosamine, and sialic acid (Table I). Only one-half of the sialic acid could be removed with *Clostridium perfringens* neuraminidase. Paper chromatography indicated that both *N*-acetylneuraminic acid (75%) and *N*-glycolylneuraminic acid (25%) were present, and

TABLE II: Amino Acid Composition[a] of Monkey Cervical Glycoprotein.

Amino Acid Residue	Cervical Glycoprotein	Alkaline Borohydride Treated
Asp	5.3	4.9
Thr	22.0	8.1
Ser	12.0	3.0
Glu	7.2	6.3
Pro	6.6	7.6
Gly	8.7	8.8
Ala	8.9	15.9
Cys/2	1.9	1.6
Val	5.4	6.7
Ile	4.3	4.7
Leu	6.5	7.0
Tyr	1.9	1.4
Phe	2.3	1.8
Lys	2.7	3.7
His	1.8	0.9
Arg	2.5	2.3
α-Aminobutyric acid		15.2

[a] Molar ratio per 100 amino residues.

the total bound sulfate ester was 1%. The two major amino acids were serine and threonine (Table II), and the total amino acid content was 19%; this value is probably lower than the correct figure since only 93% of the total composition of the glycoprotein was identified. *N*-Acetyl-D-galactosamine was shown to be linked to serine and threonine by treatment of the glycoprotein with alkaline borohydride, which gave 13% of *N*-acetyl-D-galactosaminitol, detected by gas–liquid chromatography; 23 residues of threonine and serine were converted into 22 residues of alanine and α-aminobutyric acid, respectively (Table II). This suggests that a large number of threonine and serine residues are linked by an *O*-glycosyl bond to *N*-acetyl-D-galactosamine residues.

Disulfide Bond Interactions. In order to investigate the possibility that a protein or glycoprotein may act as a bridge between the disulfide bonds of the large subunits of the mucin, the reduced and unreduced glycoproteins were analyzed by polyacrylamide gel electrophoresis (7.5% acrylamide). The glycoprotein did not penetrate the 7.5% acrylamide gel, no material staining with Coomassie blue or the HIO_4–Schiff reagent being observed inside the gels.

Circular Dichroic Studies. The effect of pH on the conformation of the glycoprotein was examined by circular dichroic measurements on solutions in 0.1 M phosphate buffer between pH 4.3 and 9.3. The spectra observed at these various pHs were identical with the spectrum given by a water solution (Figure 1), showing a characteristic negative absorption trough at 209–210 nm. Reduction with dithiothreitol and alkylation with iodoacetic acid caused a shift of the absorption trough from 209–210 to 205 nm.

Isolation and Characterization of Oligosaccharide Chains. The elution from a Bio-Gel P-2 column of the alkaline borohydride-treated glycoprotein gave fractions I (70 mg), II (240 mg), and III (25 mg) (Figure 3), for a total yield of 55% based on the glycoprotein as starting material or 65% based on the original carbohydrate content. Amino acid analysis of the acid hydrolysate of fraction I revealed the presence of all the amino acids of the original glycoprotein. As the proportions of carbohydrate components of fractions I and II are similar (Table III), it may be assumed that the carbohydrate chains of fraction I are similar to those of fraction II, but still attached to a peptide moiety. Fraction III contained less *N*-acetyl-D-galactosamine, *N*-acetyl-D-glucosamine, and sialic acid than either fraction I or II (Table III). Neither fraction II nor III contained sulfate groups, probably eliminated by the alkaline treatment.

Fraction II was further purified by paper electrophoresis in order to separate the charged from the neutral oligosaccharides (Figure 4A). The charged fraction, representing 70–80% of the material applied to the paper (18% of the staring carbohydrate material), was further fractionated by paper chromatography (Figure 4B) into three components (a,b,c). Fraction a, at the origin, did not contain *N*-acetyl-D-galactosaminitol but all the amino acids present in the original glycoprotein and represents a contamination from fraction I. Fraction b, which represented 3% of the carbohydrate content present in the original glycoprotein, had a carbohydrate composition similar to that of fraction c (Table IV), which was present in the largest proportion and represented 4% of the carbohydrate content present in the original glycoprotein. The carbohydrate compositions of fractions b and c were similar, but indicated a larger molecular weight for fraction b. Sialic acid was not determined because of insufficient material.

Fraction c was homogeneous, as observed by chromatography in two solvent systems and by electrophoresis in $Na_2B_4O_7$ buffer at pH 9.3. It exhibited human blood-group B activity, as shown by inhibition of agglutination, but no blood-group A activity, and no bound sulfate group was detected. Treatment with acid at 80 °C for 2 h released all the sialic acid residues and 82% of the L-fucose residues, and treatment for 1 and 2 h released 15% and 25%, respectively, of the D-galactose residues.

Chemical Structure of Fraction c. Periodate oxidation of fraction c degraded 1 molecule of L-fucose, 1.6 molecule of D-galactose, 1 molecule of *N*-acetyl-D-galactosaminitol, and 0.7 molecule of *N*-acetyl-D-galactosamine per average chain. The stability of 1.4 molecule of D-galactose and of 1.0 molecule of *N*-acetyl-D-glucosamine suggests the linkage of a D-galactose residue at C-3 and of the *N*-acetyl-D-glucosamine residues at C-3 or C-4. Approximately one residue of α-D-galactose could be removed by prolonged incubations of fraction c with α-galactosidase, and all the D-galactose residues were degraded by sodium periodate after removal of the α-D-galactose residue, suggesting the presence of an α-D-galactopyranosyl-(1→3)-D-galactopyranosyl residue. The methylated derivatives of fraction c indicated the presence of 2,3,4-trimethylfucitol, 2,3,4,6-tetra-*O*-methylgalactitol, and 2-deoxy-3,4,6-tri-*O*-methyl-2-(*N*-methylacetamido)galactitol. The presence of 4,6-di-*O*-methyl-D-galactose, 2,6-di-*O*-methyl-D-galactose, 2-deoxy-3,6-di-*O*-methyl-2-(*N*-methylamido)glucose, 2-deoxy-6-*O*-methyl-2-(*N*-methylamido)-glucose, and 2-deoxy-1,4-di-*O*-methyl-2-(*N*-methylamido)-galacitol was also detected.

In order to determine whether the unpurified Bio-Gel fraction II contained any unusual linkages, this fraction was methylated and gave, after hydrolysis, the methylated derivatives found in fraction c and two additional derivatives, 2,4,6-tri-*O*-methylgalactose and 3,4,6-tri-*O*-methylgalactose.

Discussion

In the present investigation, mucus was obtained from several monkeys at the time of maximum secretion. It was impossible to determine the exact phase of the cycle at which the mucus was collected since monkeys have a very irregular cycle

TABLE III: Composition of Oligosaccharides Isolated by Bio-Gel P-2 Chromatography.

Carbohydrate Residue	Fraction I		Fraction II		Fraction III	
	%	Mole Ratio[a]	%	Mole Ratio[b]	%	Mole Ratio[b]
Fuc	9	1.1	9	1.1	16	1.0
Gal	24	2.3	27	3.0	28	1.5
GalNAcol[c]			12	1.0	22	1.0
GalNAc	13	1.0	11	1.0	1	0.1
GlcNAc	16	1.3	14	1.3	2	0.1
Siac	12	0.6	20	1.0	10	0.3

[a] Relative to *N*-acetyl-D-galactosamine. [b] Relative to 2-acetamido-2-deoxy-D-galactitol. [c] Abbreviations: GalNAcol, 2-acetamido-2-deoxy-D-galactitol; Siac, sialic acid.

TABLE IV: Composition of Fraction c after Paper Chromatography.

Carbohydrate Residue	Fraction c	
	%	Mole Ratio[a]
Fuc	10	1.1
Gal	29	3.0
GalNAcol	11	1.0
GalNAc	14	1.2
GlcNAc	11	1.0

[a] Relative to 2-acetamido-2-deoxygalactitol.

in captivity. The bonnet monkey produces, per day, up to 5 g of mucus which contain approximately 97% water at mid-cycle and 93–94% of water during the luteal phase of the cycle per day (McArthur et al., 1972). Large amounts of inorganic ions (Na, Mg, K, Ca, and Zn) are also secreted at mid-cycle (Ovadia et al., 1971).

The chromatographic and electrophoretic properties of the major component of the mucus suggest that it is a single high-molecular-weight glycoprotein, which has shown to be homogeneous by equilibrium studies, N-terminal amino acid analysis, and electrophoretic analysis.

In all electrophoretic analyses with 0.5% agarose–1.5% polyacrylamide, material staining for carbohydrate did not enter the gel. Holden et al. (1971a,b) have observed the same behavior for several mucins and suggested that the accumulation of the sample at the running gel surface may be due to the gel discontinuity between the sample and the running gel.

The rather simple purification and the electrophoretic analysis of the crude monkey cervical mucus suggest that only a few contaminating proteins are present in monkey cervical mucus. A molecular mass of 10^6 daltons was calculated from the sedimentation equilibrium data. The carbohydrate moiety constitutes the major portion of the molecule (73%) and is composed of D-galactose, *N*-acetyl-D-galactosamine, *N*-acetyl-D-glucosamine, L-fucose, and sialic acid residues. The presence of D-galactose was established by reaction with D-galactose dehydrogenase whereas the D and L form of the hexosamines and hexoses was assumed. The exact ratio of *N*-acetyl- to *N*-glycolylneuraminic acid could not be determined for the whole glycoprotein since only a portion (75%) of the sialic acid could be removed with neuraminidase.

Approximately 50% of the carbohydrate–protein linkages was shown to consist of *N*-acetyl-D-galactosamine residues attached to serine or threonine residues via *O*-glycosyl bonds, 23 mol of serine and threonine per 100 mol of amino acids being involved in the carbohydrate–protein linkage. The resistance to the β elimination of some of the carbohydrate–protein linkages may be explained by the location of the serine or threonine residues in terminal position. The average amino acid residue weight of the glycoprotein was calculated to be 103 from the amino acid composition. The number of the amino acid residues in the glycoprotein (1840) was calculated from the molecular weight, the percentage of protein, and the average amino acid residue weight (103). The average number of serine and threonine residues linked to the carbohydrate moiety was 410, as shown by β elimination. The average carbohydrate residue weight was calculated to be 187 from the carbohydrate composition. From these values and from the percentage of carbohydrate, the average carbohydrate side chain was calculated to contain approximately 9–10 sugar residues. The presence of disulfide bonds in the mucins from various sources was observed by several authors (Holden et al., 1971a,b; Kim and Horowitz, 1970; Hirsch et al., 1969; Dunstone and Morgan, 1965). After cleavage of the disulfide bonds (Zahler and Cleland, 1968) with dithiothreitol, the electrophoretic mobility changed and the molecular mass was reduced from 10^6 to 4.6×10^5 daltons, indicating the presence of two large subunits identical in charge number and in size (Figure 2C). Electrophoresis in 7.5% polyacrylamide suggested that the disulfide bonds were localized between two chains of glycoprotein (glycoprotein–S–S–glycoprotein) and not between the glycoprotein and a much smaller protein or glycoprotein (glycoprotein–S–S–small glycoprotein–S–S–glycoprotein). Under the conditions of the electrophoresis, a protein or glycoprotein of less than 2500 daltons of molecular mass could have been easily detected.

The prominent feature of the circular dichroic spectra of the intact and reduced S-carboxymethylated glycoprotein was a negative trough at 209–210 nm, similar to that reported by Kabat et al. (1969) for milk oligosaccharides. This trough was shifted from 209–210 nm to 205 nm after reduction and S-carboxymethylation of the cervical mucus glycoprotein, and a concomitant increase of the negative absorption intensity beginning at 205 nm was also observed. These changes in the spectrum may be due to conformational changes of the carbohydrate or the amino acid moiety after reduction and S-carboxymethylation.

After treatment of the purified glycoprotein with alkaline borohydride, Bio-Gel P-2 chromatography, followed by paper electrophoresis and finally by paper chromatography, gave, as main components, fractions b and c which represented 3 and 4% of the carbohydrate present in the original glycoprotein. No amino acids were found in either fraction b or c. Fraction c had blood-group B activity and was homogeneous on exam-

[L-Fuc*p*-(1→2),α-D-Gal*p*-(1→3)]-D-Gal*p*-(1→4)-
D-GlcNAc*p*-(1→2,4,or6)-D-Gal*p*-(1→3or6)-D-GalNAc*p*

FIGURE 5: A partial structure for fraction c which was isolated from the cervical glycoprotein.

ination by paper chromatography in two solvent systems and by electrophoresis in borate buffer. Enzymic degradation with a purified α-D-glactosidase from ficin and periodic acid oxidation suggested that fraction c contained an α-D-galactopyranosyl-(1→3)-D-galactopyranosyl group. Mild acid treatment suggested the presence of L-fucose and sialic acid as additional terminal groups. The methylation study of fraction c suggested the presence of additional terminal residues of *N*-acetyl-D-galactosamine, of *N*-acetyl-D-galactosaminitol residues branched at C-3 and C-6, of terminal D-galactose and L-fucose residues, or a D-galactose residue branched at C-2 and C-3 and of an *N*-acetylhexosamine residue branched at C-4. From these results, a partial structure for fraction c is proposed (Figure 5). Methylation of the Bio-Gel Fraction II revealed additional methylated derivatives indicating the presence of D-galactose residues linked at C-2 and C-3, respectively. These results suggest that the structures of the carbohydrate side chains of monkey cervical glycoprotein are more complex than the structure shown in Figure 5. The disappearance of sulfate groups after the β elimination under alkaline conditions suggests a location of these groups at C-6 or C-3 of carbohydrate residues.

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