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STRUCTURE OF ACIDIC OLIGOSACCHARIDES ISOLATED FROM PRONASE-TREATED GLYCOPROTEIN OF BONNET-MONKEY (*Macaca radiata*) CERVICAL MUCUS*,†

NASIR-UD-DIN
Section of Biochemistry, Department of Chemistry, University of Baluchistan, Quetta (Pakistan)

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ABSTRACT

The major glycoprotein component of cervical mucus of bonnet monkey was treated with Pronase, and the enzyme-resistant glycoprotein purified by gel filtration on Sepharose 4B followed by DEAE-cellulose chromatography. Alkaline-borohydride cleavage of the carbohydrate chains gave a mixture of neutral and acidic oligosaccharides. Seven acidic oligosaccharides were characterized by chemical and enzymic procedures; their proposed structures are: αNeuAc(2→3)-[βGalNAc(1→4)]βGal(1→4)GlcNAc(1→6)[αFuc(1→2)βGal(1→3)]GalNAc-ol; αFuc(1→2)βGal(1→3)/6)[αNeuAc(2→3)βGal(1→4)GlcNAc(1→3)/6)]GalNAc-ol; αGalNAc(1→3)βGal(1→3)[αNeuAc(2→3)βGal(1→4)GlcNAc(1→6)]GalNAc-ol; βGlcNAc(1→3)[αFuc(1→2)]βGal(1→3)[αNeuAc(2→6)]GalNAc-ol; βGal(1→3)-[αNeuAc(2→6)]GalNAc-ol; αNeuAc(2→6)GalNAc-ol; and βGal3SO_3(1→4)GlcNAc(1→6)[αFuc(1→2)βGal(1→3)]GalNAc-ol.

INTRODUCTION

Cervical mucus is an exceedingly complex mixture of epithelial secretions, the principal constituents of which are mucin-type, carbohydrate-rich glycoproteins that share the chemical and physical properties of other epithelial secretions. The biochemical and biophysical changes in cervical mucus during the menstrual cycle influence the survival, nutrition, and passage of sperm. The cyclic alteration of the physical properties of the mucus are accompanied by variations in the carbohydrate composition[2,3] and also the chemical structure, specifically the linkage of *N*-acetylneuraminic acid, galactose, and 2-acetamido-2-deoxygalactose residues[4]. The mechanism whereby mucus secretion and glycoproteins participate in the process of reproduction is unknown. The role of glycoproteins could be assessed, because a major and a minor glycoprotein have been isolated from estrogen-stimulated cervical secretions, and antibody to the main glycoprotein[5] was raised to define the

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function of this glycoprotein[6]. In addition, salient features of the chemical structure were identified[4,7,8] that related biochemical structure to physiological function. The current study describes the structural features of the acidic oligosaccharide moiety of the main glycoprotein of the periovulatory phase cervical mucus, as purified by Pronase treatment.

EXPERIMENTAL

Collection of cervical mucus. — The cervical secretion of the bonnet monkey was collected by aspiration with a suction pump at the time of midcycle. The mucus was frozen and so maintained prior to use.

Analytical methods. — The hexose content of the glycoprotein was estimated by the phenol–sulfuric acid method[9]. Protein was assayed by absorbance at 280 nm. The neuraminic acid content was determined either by the thiobarbituric acid procedure of Warren[10] (after acid hydrolysis with 50mM sulfuric acid), or by g.l.c.

Gas–liquid chromatography. — G.l.c. determinations of the carbohydrate moiety of the glycoproteins were performed according to the procedure of Reinhold[11]. G.l.c.–m.s. of the methylated sugars was performed with a Varian MAT 731 instrument fitted with a combined c.i., e.i., and f.d. ion-source.

Column chromatography. — Columns of Bio-Gel P-200 (Bio-Rad Laboratories) and Sepharose 4B (Pharmacia Fine Chemicals) were eluted with 50mM sodium phosphate (pH 7.0) containing 0.2% sodium azide. The column of DEAE-cellulose (Whatman) was washed with 0.1M NaCl followed by a gradient of 0.1–1.0M NaCl containing 10mM HCl. Chromatography on Bio-Gel P-4 and Bio-Gel P-6 (200–400 mesh) was performed in 50mM pyridine–acetic acid (pH 5.4). The fractions containing sugar, detected by the phenol–sulfuric acid procedure and by counting tritium, were combined and lyophilized. Columns of DEAE-Sephadex A-50 were eluted with 50mM–0.5M phosphate buffer (pH 7.0) followed by 0.1–0.5M LiCl. Fractions containing carbohydrates were combined and desalted on a column of Bio-Gel P-2.

Gel electrophoresis. — Gel electrophoresis was performed as described previously[8].

Purification and Pronase treatment of the mucus glycoprotein. — The mucus glycoprotein was isolated as described earlier[7]. The lyophilized glycoprotein (99 mg) obtained after chromatography on Bio-Gel P-200 was treated with immobilized Pronase and purified on Sepharose 4B and DEAE-cellulose as described before[8]. A sodium chloride gradient was used to elute the glycoprotein from the DEAE-cellulose column. Carbohydrate- and protein-containing fractions were combined, dialyzed, and the retentate lyophilized to give the Pronase-treated glycoprotein (40 mg). The glycoprotein was examined by electrophoresis on agarose and polyacrylamide gels.

Sedimentation-equilibrium studies. — The sedimentation-equilibrium study was performed with a solution of Pronase-treated glycoprotein (1.5 mg) in one mL

of 6M guanidine hydrochloride (Heico)–0.05M tris(hydroxymethyl)aminomethane–HCl), pH 7.0, dialyzed for 48 h against the same buffer, with the meniscus-depletion sedimentation method of Yphantis[12] on a Model E ultracentrifuge. A value of 0.637 for the partial specific volume was used[13]. The molecular weight was calculated by extrapolation of the point-average molecular weights at infinite dilution.

Alkaline-borohydride treatment. — The Pronase-treated glycoprotein (30 mg) was treated with 2M sodium borohydride in 50mM sodium hydroxide according to the procedure of Iyer and Carlson[14]. A 0.2% solution of the glycoprotein in 2M sodium borohydride, containing 5 mCi of sodium [^{3}H]borohydride, in 50mM sodium hydroxide, was incubated for 18 h at 45°. After alkaline-borohydride treatment, the mixture was adjusted with 4M acetic acid to pH 5.4. The mixture was applied to a column (2.5 × 68 cm) of AG 50W-X8 (100–200 mesh) ion-exchange resin containing a 150-fold excess with respect to the sodium ions from NaOH and $NaBH_4$. Oligosaccharides, oligopeptides, and glycopeptides were eluted with water and 50mM acetic acid. Reduced oligosaccharides were separated into neutral and acidic oligosaccharides on a column (3.4 × 70 cm) of AG 1-X2 (OAc^-, 200–400 mesh) resin. The column was washed with water, 0.5M pyridine–acetic acid buffer (pH 5.4), and then with 0.1–1.5M acetic acid in the cold. The acidic oligosaccharides were further chromatographed on Bio-Gel P-6 (200–400 mesh) in 10mM pyridine–acetic acid (pH 5.4), with subsequent paper chromatography or paper electrophoresis. Paper chromatography was performed in solvents (*A*) 5:5:1:3 ethyl acetate–pyridine–acetic acid–water, and (*B*) 1:2:1 butanol–1-propanol–0.1M acetic acid. Paper electrophoresis was performed on Whatman No. 1 paper in 50mM pyridine–acetic acid, pH 5.8, for 2 h at 4°. A potential of 50 V per cm was applied.

Enzyme degradation. — Oligosaccharides were digested with some or all of the following enzymes: (*i*) α-L-fucosidase from beef epididymis (Sigma, 10mM sodium citrate buffer, pH 6.0 for 50 h at 37° and from emulsin (50mM sodium citrate buffer, pH 5.0, for 50 h at 37°; (*ii*) α-D-galactosidase from *Aspergillus niger* (Sigma, 50mM sodium citrate, pH 4.1, 70 h at 37°); (*iii*) β-D-galactosidase from *Escherichia coli* (Boehringer, 50mM sodium phosphate, pH 7.0, 48 h at 37°) and β-D-galactosidase from *Charonia lampas* (Miles, 50mM sodium citrate buffer, pH 4.0, 48 h at 37°); (*iv*) *N*-acetyl-β-glucosaminidase from jack-bean (Sigma, 50mM sodium citrate buffer, pH 4.5, 40 h at 37°); and (*v*) *N*-acetyl-α-galactosaminidase from *Charonia lampas* (Miles, 50mM sodium citrate–phosphate buffer, pH 4.1, 42 h at 37°).

Methylation analysis. — Oligosaccharides were methylated by the procedure of Hakomori[15], using iodomethane in the presence of methylsulfinyl carbanion. The methylated oligosaccharides were recovered by partition between chloroform and water, and depolymerized by treatment with 2M trifluoroacetic acid for 3 h at 105°. The cooled solution was diluted tenfold and freeze-dried. The residue in 4:1 water–methanol was treated with $NaBH_4$ (25 mg) for 12 h at 4° and for 4 h at 22°. The excess of $NaBH_4$ and sodium ions were removed simultaneously by treatment with AG 50 W-X8 (100–200 mesh) ion-exchange resin, and the reduced sugars were

TABLE I

O-METHYL DERIVATIVES OBTAINED BY ACID HYDROLYSIS OF OLIGOSACCHARIDES BEFORE AND AFTER ENZYME TREATMENT OR CHEMICAL TREATMENT

Oligosaccharides	*O-Methyl derivatives*																	
	Fuc	*Gal*						*GlcNAc*		*GalNAc*	*GalNAc-ol*				*NeuAc*		*ThrNAc-ol*	
	2,3,4-	*2,6-,*	*4,6-,*	*2,3,6-,*	*2,4,6-,*	*3,4,6-,*	*2,3,4,6-*	*3,6-,*	*3,4,6-*	*3,4,6-*	*1,4,5-,*	*1,4,5,6-,*	*1,3,4,5-*	*4,7,8,9-*	*1,4-*	
A-3	+	+		+		+		+		+	+			+		
A-3[a]	+			+		+		+		+	+					
A-3[b]		+						+		+	+			+		
A-3[c]				+			[d]	+		+	[d]	+				
A-3[e]							+	+								
A-4	+				+	+		+			+			+		
A-4[f]					+											
A-4[g]							+	+			+					
A-4[e]							+	+							+	
A-5					+			+		+	+			+		
A-5[a]					+		+	+		+	+					
A-5[g]					+		+	+			+			+		
A-5[h]					+				+	+	+					
A-6	+	+							+		+			+		
A-6[a]	+	+							+			+				
A-6[f]					+				+		+			+		
A-7							+				+			+		
A-7[a]							+					+				
A-8													+	+		
S-1	+				+	+		+			+					
S-1[c]	+					+	+	+			+					
S-1[b,i]							[d]		+		[d]		+			

[a]Neuraminidase-treated. [b]α-L-Fucosidase- and β-D-galactosidase-treated. [c]Sequentially neuraminidase-, α-fucosidase- and β-galactosidase-treated. [d]Low proportion. [e]Periodate degraded. [f]α-1-Fucosidase-treated. [g]*N*-acetyl-α-D-galactosaminidase-treated. [h]Neuraminidase- and β-D-galactosidase-treated. [i]-Desulfated oligosaccharide.

eluted with water, methanol, and methanolic NH_3. The combined washings were evaporated, and the residue in methanol was repeatedly evaporated to remove boric acid. Finally, the residue was acetylated with pyridine (0.5 mL) and acetic anhydride (0.4 mL) for 12 h at 22° and the methylated alditols were examined by g.l.c. and g.l.c.–m.s. (see Table I).

Periodate oxidation–sodium borohydride reduction. — Reduced oligosaccharides (1–3 mg) were oxidized with 0.1M periodate for 12 h at 4° and then for 8 h at room temperature. The reaction was processed conventionally and the products treated with $NaBH_4$ (25 mg/mg oligosaccharide for 12 h at 4° followed by 5 mg/mg for 6 h at temperature). Sodium ions were removed with AG 50W-X8 (100–200 mesh) ion-exchange resin and boric acid by evaporation with methanol. The residue was treated with 0.25M H_2SO_4 for 2.5 h and deionized with AG 1-X8 (OAc^-, 100–200 mesh) resin. After methanolysis with 0.5M methanolic hydrogen chloride for 20 h at 80°, the products were examined by g.l.c. in comparison with reference standards. Samples of 2-acetamido-2-deoxythreitol, 2-acetamido-2-deoxyarabinitol, and *N*-acetylserinol were obtained by periodate oxidation (10mM) of 2-acetamido-2-deoxy-*O*-galactitol for 30 min at 22°.

Removal of sialic acid. — Sialic acid was removed from oligosaccharides (2–3 mg) by treatment with neuraminidase (*Vibrio cholerae,* 100–200 μL; 50–100 units, Behring Diagnostics) in 0.1% $CaCl_2$ and 0.5% NaCl, pH 5.5, for 24 h at 37°. The reaction was terminated by immersing the vessel in a boiling-water bath for 3 min. The mixture was chromatographed and the eluates containing oligosaccharides were combined and lyophilized. Neuraminidase-treated oligosaccharides were methylated, and the methylated sugars were identified as already described.

RESULTS

Purification and characterization of Pronase-degraded cervical mucus glycoprotein. — The mucus glycoproteins obtained from bonnet monkeys at the periovulatory phase of the menstrual cycle were purified by gel filtration on Bio-Gel P-200 followed by treatment with Pronase. Pronase-treated glycoproteins were fractionated on Sepharose 4B. The main fraction (80%) showed in DEAE-cellulose chromatography the presence of a single glycoprotein (Fig. 1). The glycoprotein, in polyacrylamide gel electrophoresis, did not enter the gel, and no contaminating proteins or glycoproteins were observed. In agarose (1%), the glycoprotein entered the gel and exhibited a single component. The purity of the glycoprotein was further assessed by sedimentation equilibrium; a molecular mass of 1×10^5 was calculated. The carbohydrate and amino acid composition, and the sulfate content of the glycoprotein are given in Table II.

Preparation of acidic oligosaccharide-alditols. — The glycoprotein was subjected to reductive cleavage by alkaline borohydride yielding a mixture of oligosaccharide-alditols. A decrease of serine and threonine and a corresponding increase of alanine and the appearance of 2-aminobutanoic acid were detected in

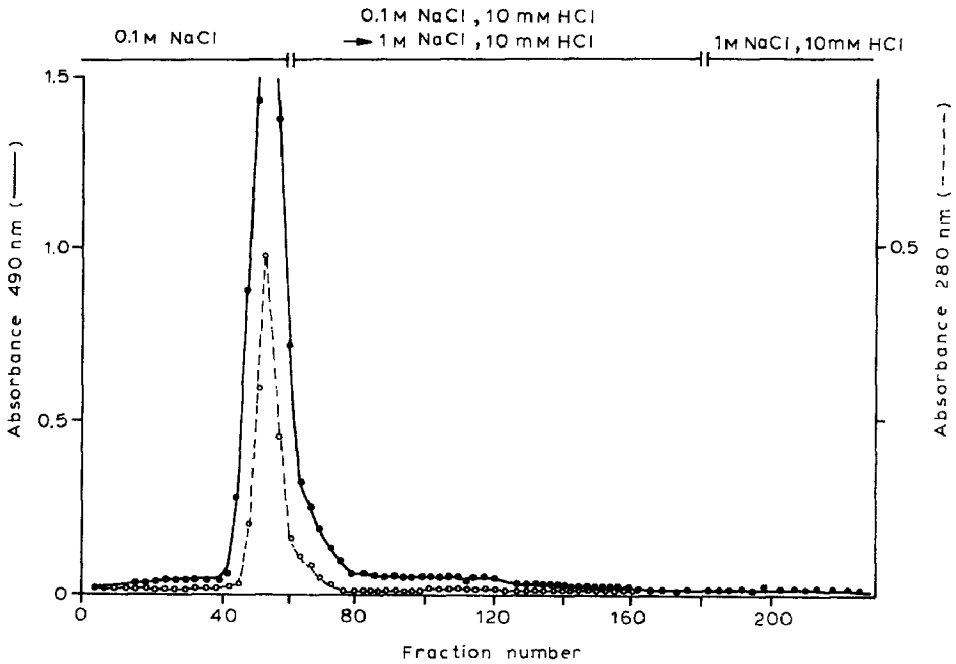

Fig. 1. DEAE-cellulose chromatography of Pronase-treated major glycoprotein fraction from Sepharose 4B. Fractions of 3 mL were collected and every third fraction was examined for hexoses by phenol–sulfuric acid and for amino acids by absorbance at 280 nm.

hydrolyzates of the protein. The acidic oligosaccharides eluted from the column of AG 1-X2 were separated on a column of Bio-Gel P-6 into seven fractions (Table III, Fig. 2). The acetic acid (0.1–0.5M) washing from the column of AG 1-X2 afforded an acidic oligosaccharide (S-1) that was purified on a cellulose plate (Table III). Fractions A-1 and A-2 constituted mainly glycopeptides, as indicated by the low percentage of 2-acetamido-2-deoxygalactitol and the presence of hydroxylated amino acids.

Desulfation of oligosaccharides. — The oligosaccharides (0.3–0.7 mg) were desulfated according to the procedure of Kantor and Schubert[16].

Oligosaccharide fraction A-3. — This fraction was further purified by chromatography on DEAE-Sephadex A-50 and was homogeneous in paper chromatography in solvents *A* and *B*. Its sequential treatment with neuraminidase, α-L-fucosidase, and with β-D-galactosidase removed residues of fucose, galactose, and *N*-acetylneuraminic acid. Methylation of the residual oligosaccharide showed the presence of terminal *N*-acetylgalactosamine, a terminal galactose (small proportion), 4-linked galactose, 4-linked *N*-acetylglucosamine, and 6-linked and 3,6-linked (small proportion) 2-acetamido-2-deoxygalactitol. These results suggest incomplete removal of a galactose residue. Incomplete removal of terminal galactose linked to 2-acetamido-2-deoxygalactitol that is substituted by a chain at

TABLE II

CARBOHYDRATE AND AMINO ACID COMPOSITION OF GLYCOPROTEIN PURIFIED ON DEAE-CELLULOSE

	Percentage	*Molar ratio*[a]
Carbohydrate		
L-Fucose	8	0.83
D-Galactose	21	2.00
N-Acetylglucosamine	13	1.00
N-Acetylgalactosamine	19	1.46
N-Acetylneuraminic acid	11	0.60
Sulfate	1.3	—
Total carbohydrate	72	—
Amino acids[b]		
Aspartic acid	44	
Threonine	210	
Serine	99	
Glutamic acid	84	
Proline	88	
Glycine	71	
Alanine	86	
Cystine/2	11	
Valine	72	
Methione	—	
Leucine	61	
Isoleucine	48	
Tyrosine	29	
Phenylalanine	31	
Lysine	32	
Histidine	16	
Arginine	18	

[a]Molar ratio relative to *N*-acetylglucosamine. [b]Residues per 1000 residues.

TABLE III

CARBOHYDRATE COMPOSITION OF PURIFIED OLIGOSACCHARIDES

Oligo-saccharides	*Fucose*		*Galactose*		N-*Acetyl glucosamine*		N-*Acetyl galactosamine*		N-*Acetyl neuraminic acid*		*2-Acetamido-2-deoxy-galactitol*	
	%	*MR*[a]	%	*MR*[a]	%	*MR*[a]	%	*MR*[a]	%	*MR*[a]	%	*MR*[a]
A-3	8	0.67	22	1.708	13	0.81	14	0.88	19	0.85	16	1.00
A-4	8	0.54	26	1.60	15	0.75			24	0.86	20	1.00
A-5			24	1.65	15	0.83	14	0.78	20	0.80	18	1.00
A-6	12	0.77	15	0.88	17	0.81			26	0.89	21	1.00
A-7			18	0.71					40	0.92	31	1.00
A-8									47	0.80	42	1.00
S-1[b]	10	0.56	30	1.61	18	0.75					24	1.00

[a]Molar ratio relative to 2-acetamido-2-deoxygalactitol. [b]Sulfated oligosaccharide. Sulfate not determined.

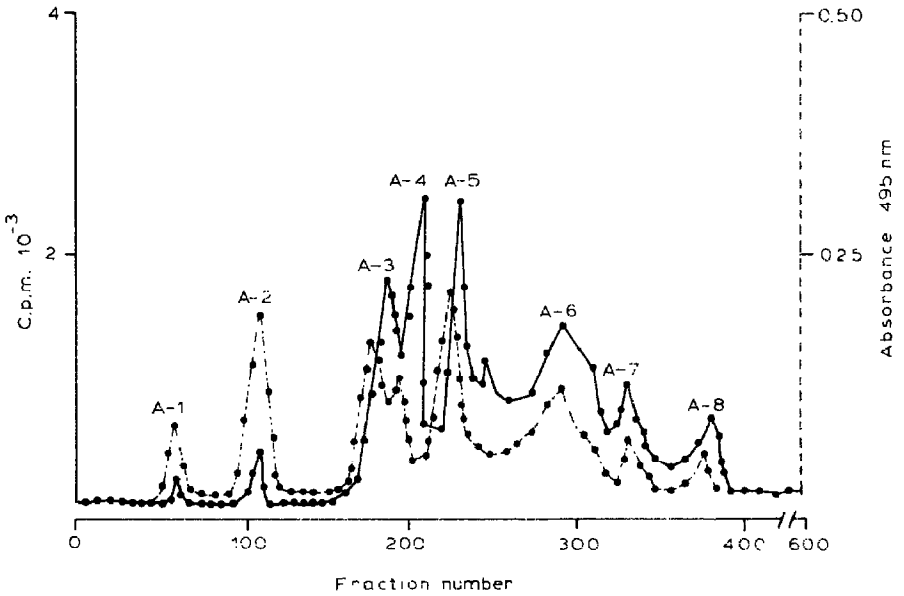

Fig. 2. Separation of oligosaccharides on a column of Bio-Gel P-6. Eight fractions (A-1, 2 mg; A-2, 4 mg; A-3, 6 mg; A-4, 7 mg; A-5, 8 mg; A-6, 4 mg; A-7, 3 mg; and A-8, 2 mg) were obtained by elution with 50mM pyridine–acetic acid. Hexoses were monitored by the phenol-sulfuric acid assay.

O-6 has been observed[17]. Methylation of the oligosaccharide after treatment with neuraminidase alone showed the presence of terminal *N*-acetylgalactosamine, terminal fucose, 4-linked and 2-linked galactose, 4-linked *N*-acetylglucosamine, and 3,6-linked *N*-acetylgalactosaminitol; these results suggest that sialic acid is linked to O-3 of a galactose residue. Methylation of the oligosaccharides after sequential treatment with α-L-fucosidase and β-D-galactosidase showed the presence of 3,4-linked galactose, 4-linked *N*-acetylglucosamine, terminal *N*-acetylgalactosamine, and 6-linked 2-acetamido-2-deoxygalactitol. These results suggest that fucose is linked to O-2 of a galactose residue that is adjacent to 2-acetamido-2-deoxygalactitol. Treatment of the oligosaccharide with α-*N*-acetylgalactosaminidase did not remove *N*-acetylgalactosamine.

Periodate oxidation–borohydride reduction followed by methylation of the degraded oligosaccharide showed the presence of a terminal galactose group and 4-linked *N*-acetylglucosamine.

Methylation of the oligosaccharide showed the presence of terminal *N*-acetylgalactosamine, sialic acid, and fucose groups; 3,4-linked and 2-linked galactose; 4-linked *N*-acetylglucosamine, and 3,6-linked 2-acetamido-2-deoxygalactitol. The results of these experiments showed that oligosaccharide A-3 is a heptasaccharide having the sequence, linkages and anomeric configurations as shown in Fig. 3.

Oligosaccharide fraction A-4. — Oligosaccharide A-4 was purified on DEAE-Sephadex A-50 and was homogeneous in p.c. (solvents *A* and *B*) and in p.e.

Periodate oxidation–borohydride reduction completely removed fucose,

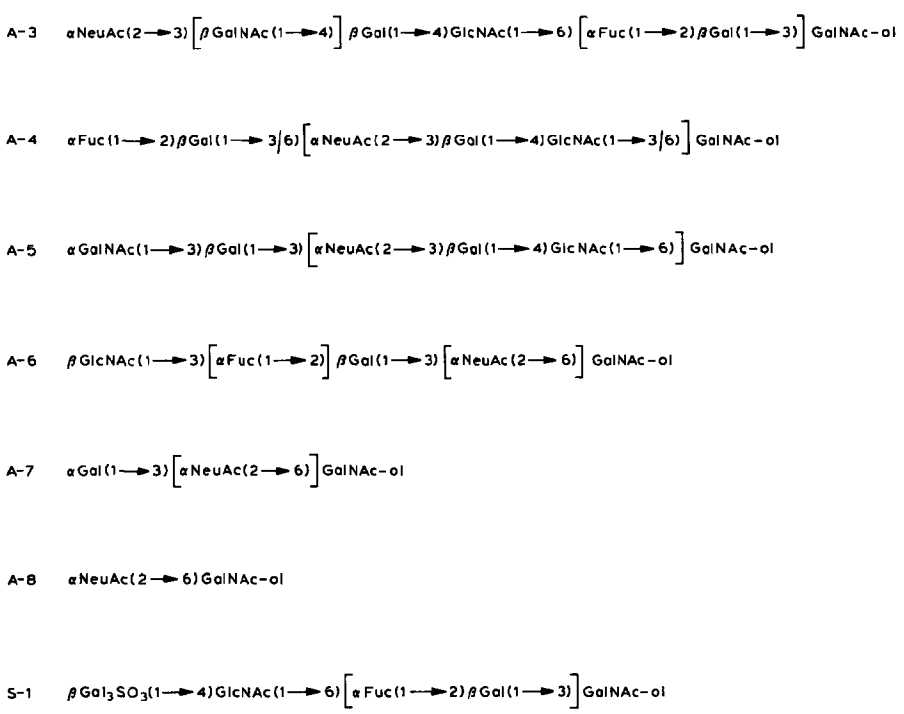

Fig. 3. Proposed structure for acidic oligosaccharides.

sialic acid, and a galactose residue, and converted 2-acetamido-2-deoxygalactitol into 2-acetamido-2-deoxythreitol. Methylation of the sugar components of the degraded oligosaccharide showed the presence of terminal galactose, 4-linked *N*-acetylglucosamine, and 3-linked 2-acetamido-2-deoxythreitol. Sequential treatment of oligosaccharide with α-L-fucosidase and β-D-galactosidase (*C. lampas*) showed the loss of a fucose and a galactose residue. Methylation of the oligosaccharide after treatment with α-L-fucosidase showed the presence of terminal galactose and *N*-acetylneuraminic acid, 3-linked galactose, 4-linked *N*-acetylglucosamine, and 3,6-linked 2-acetamido-2-deoxygalactitol. Methylation of the sequentially α-L-fucosidase- and neuraminidase-treated oligosaccharide showed the presence of terminal galactose, 4-linked *N*-acetylglucosamine, and 3,6-linked 2-acetamido-2-deoxygalactitol. Methylation of the native oligosaccharide showed the presence of terminal neuraminic acid and fucose, 3-linked and 2-linked galactose, 4-linked *N*-acetylglucosamine, and 3,6-linked 2-acetamido-2-deoxygalactitol. From these studies the linkages, sequences, and anomeric configurations of the sugar residues present in the oligosaccharide is defined. However, the identity of the sugars that are linked to 2-acetamido-2-deoxygalactitol at O-3 and O-6 is not clear. It is possible that periodate oxidation, which was performed at 4°, was not complete or that oligosaccharide may be a mixture of two similar components differing only in substitution on 2-acetamido-2-deoxygalactitol. Based on these results, the structure shown in Fig. 3 is proposed for oligosaccharide A-4.

Oligosaccharide fraction A-5. — Methylation of the oligosaccharide showed the presence of terminal neuraminic acid and *N*-acetylgalactosamine, 3-linked galactose, 4-linked *N*-acetylglucosamine, and 3,6-linked 2-acetamido-2-deoxygalactitol. Periodate oxidation and borohydride reduction resulted in total decomposition of *N*-acetylgalactosamine and sialic acid, and conversion of 2-acetamido-2-deoxygalactitol into 2-acetamido-2-deoxythreitol. Galactose and *N*-acetylglucosamine were recovered unchanged.

Treatment with neuraminidase followed by methylation of the residual oligosaccharide and identification of methylated sugars showed the presence of terminal galactose, 3-linked galactose, terminal *N*-acetylgalactosamine, 4-linked *N*-acetylglucosamine and 3,6-linked 2-acetamido-2-deoxygalactitol, suggesting that neuraminic acid is linked to O-3 of a galactose residue.

Treatment of the oligosaccharide with *N*-acetyl-α-galactosaminidase and subsequent methylation of the residual oligosaccharide showed that the *N*-acetylgalactosamine is linked to O-3 of a galactose residue. Sequential treatment of the oligosaccharide with neuraminidase and β-D-galactosidase, and subsequent methylation of the residual oligosaccharide, showed the presence of terminal *N*-acetylglucosamine and *N*-acetylgalactosamine, 3-linked galactose, and 3,6-linked 2-acetamido-2-deoxygalactitol. These results suggest the structure of oligosaccharide A-5 shown in Fig. 3.

Oligosaccharide fraction A-6. — The oligosaccharide A-6 was purified by p.c. in solvent *A* followed by chromatography on DEAE-Sephadex A-50, and was homogeneous in p.c. in solvents *A* and *B*. Periodate oxidation–borohydride reduction decomposed fucose, *N*-acetylglucosamine, and neuraminic acid, and converted 2-acetamido-2-deoxygalactitol into 2-acetamido-2-deoxythreitol: galactose was recovered unchanged. Methylation of the oligosaccharide showed the presence of terminal fucose, sialic acid, and *N*-acetylglucosamine, 2,3-linked galactose, and 3,6-linked 2-acetamido-2-deoxygalactitol. Treatment of the oligosaccharide with α-L-fucosidase and subsequent methylation of the residual oligosaccharide showed the presence of terminal *N*-acetylglucosamine and neuraminic acid, 3-linked galactose, and 3,6-linked 2-acetamido-2-deoxygalactitol. Treatment of the oligosaccharide with neuraminidase followed by methylation showed the presence of terminal fucose and *N*-acetylglucosamine, 2,3-linked galactose, and 3-linked 2-acetamido-2-deoxygalactitol. These results provide evidence that oligosaccharide A-6 is a pentasaccharide with the structure shown in Fig. 3.

Oligosaccharide fraction A-7. — The oligosaccharide was purified on Bio-Gel P-6 and was a single component in p.c. in solvents *A* and *B*. Methylation of the oligosaccharide and identification of the products showed terminal galactose and neuraminic acid, and 3,6-linked 2-acetamido-2-deoxygalactitol. Treatment of the oligosaccharide with neuraminidase and subsequent methylation of the remaining oligosaccharide showed that sialic acid was linked to O-6 of 2-acetamido-2-deoxygalactitol. These data suggest the structure of oligosaccharide A-7 as shown in Fig. 3.

Oligosaccharide fraction A-8. — Methylation of the oligosaccharide and identification of the product showed the presence of a terminal sialic acid and 6-linked 2-acetamido-2-deoxygalactitol. These data suggest the structure shown in Fig. 3 for oligosaccharide A-8.

Oligosaccharide fraction S-1. — This fraction, obtained by washing the AG-1 column with acetic acid, was purified on cellulose plates in solvent *A*: it was obtained in very small amount and underwent degradation at room temperature. The carbohydrate composition of the oligosaccharide did not change after desulfation. Methylation of the desulfated oligosaccharide and identification of the methylated sugars showed therein the presence of terminal galactose and fucose, 2-linked galactose, 4-linked *N*-acetylglucosamine, and 3,6-linked 2-acetamido-2-deoxygalactitol. Methylation of the intact oligosaccharide and identification of the methylated sugars showed the presence of terminal fucose, 2-linked and 3-linked galactose residues, 4-linked *N*-acetylglucosamine and 3,6-linked 2-acetamido-2-deoxygalactitol. Sequential treatment of the desulfated oligosaccharide with α-D-galactosidase and α-L-fucosidase removed a fucose residue. Treatment of the desulfated oligosaccharide with α-L-fucosidase and β-D-galactosidase removed a fucose and partially removed two galactose residues. Methylation of the α-L-fucosidase- and β-D-galactosidase-treated oligosaccharide showed the presence of terminal *N*-acetylglucosamine, a small amount of terminal galactose, and 6-linked and 3,6-linked (small amounts) 2-acetamido-2-deoxygalactitol. The presence of terminal galactose and 3,6-linked 2-acetamido-2-deoxygalactitol in the products of methylation, after treatment with α-L-fucosidase and β-D-galactosidase, suggests that β-galactosidase did not remove the galactose residues completely. It is known that galactose residues linked to 2-acetamido-2-deoxygalactitol, bearing a chain at O-6, are not removed completely by β-D-galactosidase[17]. It is, therefore, unlikely that the oligosaccharide was impure; evidently the glycosidase did not remove hexose completely. As a consequence, terminal galactose and 3,6-linked 2-acetamido-2-deoxygalactitol were observed. Based on these results, the structure shown in Fig. 3 is proposed for oligosaccharide S-1.

DISCUSSION

The separation of secreted glycoprotein from the remaining polymeric materials, namely serum glycoproteins, proteins, and enzymes, was readily accomplished by gel filtration on Bio-Gel P-200. The glycoprotein component from the column of Bio-Gel P-200 was treated with Pronase to remove any contaminating proteins and glycoproteins. It is known that proteolytic enzymes degrade a minor glycoprotein component of cervical mucus glycoproteins[8], and remove the hydrophobic-binding region from the protein core[18]. Fractionation of Pronase-treated glycoprotein on Sepharose 4B resulted in two fractions. The major fraction in ion-exchange chromatography resulted in a single component of high molecular weight. Despite the fact that glycoproteins are known to be degraded by Pronase[8,18], a

high-molecular-weight (1×10^5) glycoprotein was obtained that was homogeneous, albeit polydisperse, in agarose electrophoresis and free of contaminating proteins. Sialic acid in the glycoprotein was present as *N*-acetylneuraminic acid. This is similar to observations with human cervical mucus[19]; bovine material contains *N*-glycosylneuraminic acid[20]. The glycoprotein was free of the cross-linked protein fractions containing cystine that are, observed with bovine cervical mucus[21].

Treatment of the glycoprotein with alkaline borohydride gave a mixture of oligosaccharides that was fractionated on Bio Gel P-6. The saccharides obtained from Bio-Gel P-6 were further purified by ion-exchange and paper chromatography, affording six sialylated oligosaccharides. A sulfated oligosaccharide was obtained by washing the AG-1 column with acid. The oligosaccharides characterized may be considered to have the following core structures:

(i) βGal(1→3)GalNAc-ol
(ii) βGal(1→4)βGlcNAc(1→6)GalNAc-ol

Chain elongation on these two core residues takes place, resulting in the structures here characterized and shown in Fig. 3. The partial structures of few carbohydrate chains have been identified for human cervical glycoprotein[17], and these bear similarity to the core structures of the bonnet-monkey oligosaccharides. Monosialylated oligosaccharides from bonnet-monkey cervical glycoprotein obtained at midcycle have been characterized by n.m.r. spectroscopy[22]. These structures, like those of human cervical oligosaccharides, have common core-residues and also share some chain structures.

A variety of carbohydrate chain-lengths in mucins[23,24] and in blood group-active glycoproteins[25–27] is known, and the heterogeneity of cervical mucus glycoprotein could be even wider because of changing physicochemical behavior with the menstrual cycle.

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