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Note

Changes in the glycoprotein structure of the cervical mucus of the bonnet monkey during the menstrual cycle. Study of the premenstrual-phase mucus*

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Cervical mucus is a hydrophilic, gel-like, dynamic epithelial secretion, which implements an important role in the mammalian reproductive cycle. It displays distinct biophysical and morphological variations during the menstrual cycle. The mucus glycoproteins, which constitute the totality of the macromolecular components of the mucus, exhibit alterations of their carbohydrate composition during the menstrual cycle[4,5]. An investigation of the chemical structure of the mucus glycoproteins was undertaken in order to understand their contribution to the reproductive cycle and relate changes in their chemical structure to the biophysical properties of the mucus in various phases of the menstrual cycle. The cervical mucus of the bonnet monkey was chosen for this study, as it is produced in large amounts, and the menstrual cycle of the bonnet monkey is very similar to that of the human[6].

The glycoprotein component of the premenstrual-phase cervical mucus was purified[3] (for composition, see Table I) and fractionated, as previously described for the peri-ovulatory phase mucus[7]. The oligosaccharides from the major glycoprotein fraction were prepared by reductive β-elimination, as described by Iyer and Carlson[8]. After separation on a column of Bio-Gel P-4 (Fig. 1), the main oligosaccharide fraction (Fraction B) was obtained in 62% yield, and shown to be homo-

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geneous by paper electrophoresis in borate buffer and by paper chromatography in two solvent systems. On further purification by ion-exchange chromatography, it gave a single, carbohydrate-containing fraction (Fig. 2, Fraction B-1) in 86% yield (see Table I). This fraction was shown to be homogeneous by paper chromatography in two solvent systems, as well as by the symmetrical appearance of the elution peak. The fraction constitutes ~65, 60, 50, 60, and 55% of the 2-acetamido-2-deoxy-D-galactose, 2-acetamido-2-deoxy-D-glucose, *N*-acetylneuraminic acid, L-fucose, and D-galactose residues, respectively, present in the original glycoprotein. These proportions suggest either a slight loss of *N*-acetylneuraminic acid residues during the purification procedure, or some elimination of oligosaccharides containing these residues. The proportion of 2-acetamido-2-deoxy-D-galactitol indicates the presence of a heptasaccharide or a mixture of hexa-, hepta-, and octa-saccharides, as oligosaccharides of lower mol. wt. could be separated distinctly by the chromatographic procedures used.

Methylation studies of Fraction B-1 (see Table II) showed the presence of terminal L-fucopyranosyl residues; of terminal, 2-linked, and 6- and 3-linked D-galactopyranose residues in the relative proportions of 1:1:1; of 4-linked 2-acetamido-2-deoxy-D-glucopyranose residues; and of 6-linked, and 3- and 6-linked 2-acetamido-2-deoxy-D-galactopyranose residues, in the proportion of 2:1.

Mild acid-treatment of the methylated oligosaccharide with 50mM sulfuric acid for 75 min at 80° removed ~70% of the terminal *N*-acetylneuraminic acid residues. Subsequent trideuteriomethylation introduced a trideuteriomethoxyl group at C-3 of the D-galactopyranose residues linked at O-6, and (in very small proportion) at C-3 of the terminal D-galactopyranose residues. The incorporation of a trideuteriomethoxyl group at C-3 of terminal D-galactopyranose residues arising from partial removal of terminal L-fucopyranose residues is unlikely, as the ratio of L-fucose to terminal D-galactose before and after acid treatment was very similar. The incorporation of the trideuteriomethoxyl group at only C-3 of the D-galactopyranose residues is also indicative of the selectivity of the cleavage of sialic acid residues.

For the Smith degradation of Fraction B-1, the periodate oxidation was conducted for 16 h at 22°. The oxidized fragments were reduced with sodium borohydride, and the resulting polyalcohols were hydrolyzed by mild acid-treatment. The results of the degradation are shown in Table I. As expected from the results of the methylation study, *N*-acetylneuraminic acid, 2-acetamido-2-deoxy-D-galactose, and L-fucose residues were completely degraded, as well as ~50% of the D-galactose residues. The 2-acetamido-2-deoxy-D-galactitol residues were converted into 2-acetamido-2-deoxy-D-threitol and 2-acetamido-2-deoxyglycerol residues. These results suggest that the oligosaccharide fraction has a considerable proportion of D-galactose residues, in addition to L-fucose, 2-acetamido-2-deoxy-D-galactose, and *N*-acetylneuraminic acid residues, as nonreducing, terminal sugars. The formation of 2-acetamido-2-deoxy-D-threitol by Smith degradation suggests that nearly two-fifth of the 2-acetamido-2-deoxy-D-galactose residues linked to the protein core are substituted at both O-3 and O-6, or at O-3 alone.

TABLE I

COMPOSITIONS OF ORIGINAL GLYCOPROTEIN; OF OLIGOSACCHARIDE FRACTIONS A, B, AND C OBTAINED FROM A COLUMN OF BIO-GEL P-4; OF FRACTION B-1 OBTAINED FROM A COLUMN OF DEAE-SEPHADEX; AND OF FRACTION SB-1 OBTAINED BY SMITH DEGRADATION

Components[a]	*Original*		*Fractions*								
			A		*B*		*C*		*B-1*		*SB-1*
	%	*M.r.*[b]	%	*M.r.*[b]	%	*M.r.*[b]	%	*M.r.*[b]	%	*M.r.*[b]	*M.r.*[b]
2-Acetamido-2-deoxy-D-galactitol	0.0	0.00	1.0	0.06	14.5	0.96	16.6	1.18	14.5	0.96	0.00
2-Acetamido-2-deoxy-D-galactose	14.0	1.58	15.7	1.02	11.0	0.73	12.4	0.89	11.2	0.73	0.00
2-Acetamido-2-deoxy-D-glucose	9.0	1.00	15.4	1.00	15.0	1.00	14.0	1.00	15.0	1.00	1.00
2-Acetamido-2-deoxy-glycerol											0.60
2-Acetamido-2-deoxy-D-threitol											+[c]
N-Acetylneuraminic Acid	10.0	0.79	7.0	0.36	11.0	0.52	3.7	0.19	14.2	0.68	0.00
L-Fucose	6.0	0.90	8.0	0.70	8.5	0.76	8.8	0.84	10.0	0.90	0.00
D-Galactose	16.0	2.18	21.0	1.67	25.0	2.05	26.0	2.23	25.0	2.03	1.00

[a]Determined by g.l.c. [b]Molar ratio relative to 2-acetamido-2-deoxy-D-glucose. [c]Present but not determined.

The formation of 2-acetamido-2-deoxyglycerol by the same degradation-method suggests that the remaining 2-acetamido-2-deoxy-D-galactose residues linked to the protein core are either substituted at O-6, or are unsubstituted. The methylation results (Table II), however, show that these residues are substituted at O-6, and at

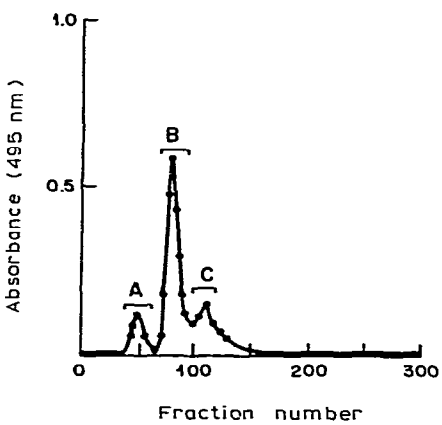

Fig. 1. Elution profile of oligosaccharides from a column of Bio-Gel P-4. The column was eluted with 50mM pyridine–acetic acid (pH 5.4, 750 mL), and fractions of 2.5 mL were collected. Every third fraction was examined for the presence of carbohydrates by the phenol–sulfuric acid method[11]. Fraction A: tubes 39–63; Fraction B: tubes 70–93; and Fraction C: tubes 102–120.

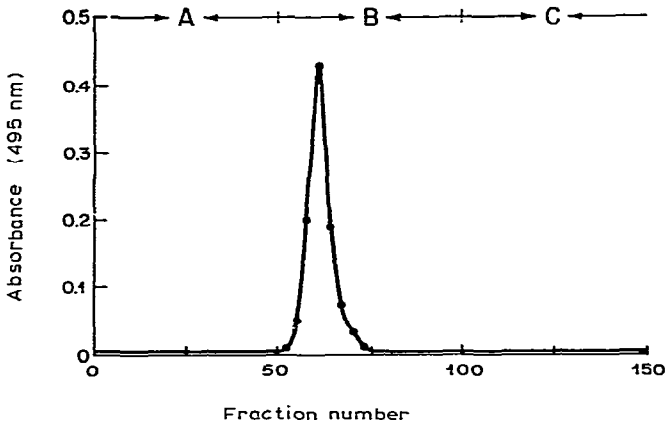

Fig. 2. Elution pattern of Fraction B from a column of DEAE-Sephadex A-25. The column was eluted with: (A) 50mM sodium monophosphate (pH 6.0, 150 mL); (B) a gradient of 0.05–0.5M lithium chloride in 50mM sodium monophosphate (pH 6.0, 150 mL); and (C) 0.5M lithium chloride in 50mM sodium monophosphate (pH 6.0, 150 mL). Fractions of 3 mL were collected, and every third fraction was examined for hexoses by the phenol–sulfuric acid procedure[11]. Fractions 50–75 were combined to give Fraction B-1.

O-3 and O-6. The resistance of the 2-acetamido-2-deoxy-D-glucose residues to periodate oxidation is consistent with the substitution at O-4 established by the methylation studies.

Structural study of the oligosaccharides obtained by degradation of the peri-ovulatory-phase glycoprotein has established that β-D-galactopyranose residues are substituted at O-3 by α-D-galactopyranose and at O-2 by L-fucopyranose, terminal residues[3], or at O-6 by 2-acetamido-2-deoxy-D-galactopyranose and at O-2 by L-fucopyranose terminal residues[7]. In addition, the 2-acetamido-2-deoxy-D-galactose residues linked to the serine or threonine residues of the protein backbone are substituted at O-3 by 2-acetamido-2-deoxy-D-glucopyranose chain residues and at O-6 by terminal residues of α-*N*-acetylneuraminic acid[9]. In contrast, the results of the methylation and periodate-oxidation studies of the pre-menstrual, main glycoprotein described in this article suggest that D-galactopyranose residues are substituted at O-6 (or O-2) by L-fucopyranose, D-galactopyranose, or 2-acetamido-2-deoxy-D-galactopyranose terminal residues and at O-3 by terminal residues of *N*-acetylneuraminic acid; and the 2-acetamido-2-deoxy-α-D-galactopyranose residues linked to serine (or threonine) are substituted at O-6 by a chain residue of 2-acetamido-2-deoxy-D-glucopyranose and at O-3 by terminal residues of L-fucopyranose, D-galactopyranose, or 2-acetamido-2-deoxy-D-galactopyranose. Thus, the major glycoproteins obtained from mucus of the peri-ovulatory and of the pre-menstrual phases differ mainly in the point of linkage of the *N*-acetylneuraminic acid terminal residues and of the substituents of the 2-acetamido-2-deoxy-D-galactopyranose residues linked to the protein chain. In addition, the glycoprotein component from the cervical mucus of the pre-menstrual phase differs from that of the peri-ovulatory

phase in being more heterogeneous and in containing several glycoproteins having different *N*-terminal amino acid residues[9].

It is not known, as yet, whether changes observed during the menstrual cycle are restricted only to the linkages of the carbohydrate residues or also occur in the protein backbone. It is also unclear as to whether the diversity in the carbohydrate structures arises from changes within the same glycoprotein macromolecule or the production of two or more different glycoproteins.

EXPERIMENTAL

Analytical methods. — Gas–liquid chromatography of reducing and non-reducing sugars was performed according to the procedure of Reinhold[10] with a Perkin–Elmer 900 gas chromatograph equipped with a dual-ionization detector. G.l.c.–mass spectrometry of the methylated sugars was performed with an analytical system consisting of an IBM-1800 computer fed with raw data generated by a single-focusing, Hitachi–Perkin–Elmer RMU-6 mass spectrometer interfaced with a Perkin–Elmer 900 gas chromatograph. The hexose content of the eluates from the columns of Bio-Gel P-4, AG 50W-X8, and DEAE-Sephadex was determined by the phenol–sulfuric acid method[11].

Preparation of oligosaccharides from pre-menstrual mucus glycoprotein. — The cervical mucus was collected (by aspiration with a suction pump) at times other than the mid-cycle estrogen surge. It was fractionated on columns of Bio-Gel P-200 and Sepharose 2B, as described for the preparation of peri-ovulatory glycoproteins[3,7]. The major glycoprotein (50 mg; for composition, see Table I), obtained from the Sepharose 2B column, was subjected to reductive β-elimination under conditions similar to those described by Iyer and Carlson[8]. After completion of the reaction, the pH of the solution was adjusted to 5.0 with 4M acetic acid and the solution applied to a column (2.2 × 60 cm) of AG 50W-X8 (H^+, 100–200 mesh) ion-exchange resin. The column was eluted with water (300 mL), and then with 10mM acetic acid (250 mL). The combined eluates were lyophilized, and the remaining boric acid was removed from the residue by repeated addition and evaporation of methanol. The residue (37 mg) was applied to a column (2.2 × 166 cm) of Bio-Gel P-4 (200–400 mesh), and the column was eluted with 50mM pyridine–acetic acid (pH 5.4, 750 mL). The carbohydrate-containing fractions (see Fig. 1) were combined and lyophilized to give Fraction A (6 mg), Fraction B (23 mg), and Fraction C (5 mg) (for composition, see Table I). Electrophoresis of Fraction B in 50mM sodium borate buffer (pH 9.5) at 1500V for 2 h, and staining with periodate–benzidine[12] (after spraying with 6M acetic acid) revealed a single, negatively charged component (2.8 cm). Paper chromatography in 1-butanol–pyridine–water (5:1:4, v/v, upper layer, Solvent A, $R_{Cellobiose}$ 0.13) and 1-butanol–propanol–0.1M hydrochloric acid (1:2:1, Solvent B, $R_{Cellobiose}$ 0.30) showed the presence of a periodate–benzidine-positive component with minimal tailing. A solution of the main oligosaccharide fraction (Fraction B, 21 mg) in 50mM sodium phosphate buffer (pH 6.0, 1 mL) was applied to a column

(1.5 × 40 cm) of DEAE-Sephadex A-25. The column was eluted with 50mM sodium phosphate (pH 6.0, 150 mL), and then with a gradient of 0.05–0.5M lithium chloride in 50mM sodium phosphate (pH 6.0, 150 mL), followed by 0.5M lithium chloride in 50mM sodium phosphate (pH 6.0, 150 mL). The carbohydrate-containing fractions (see Fig. 2) were combined, evaporated, and rechromatographed on a column (2.2 × 38 cm) of Bio-Gel P-2. The carbohydrate-containing fractions were combined and lyophilized to give Fraction B-1 (18 mg; for composition, see Table I). Fraction B-1, on chromatography in solvents A ($R_{Cellobiose}$ 0.15) and B ($R_{Cellobiose}$ 0.32), showed the presence of a single component.

Methylation of oligosaccharide Fraction B-1. — A portion of Fraction B-1 (10 mg) was methylated[13] in dimethyl sulfoxide (1 mL) with methylsulfinylsodium (1 mL) for 2 h at 22°, with subsequent addition of methyl iodide (0.5 mL). The solution was stirred for 3 h, and then diluted with water (25 mL) and lyophilized. The residue was extracted with 9:1 (v/v) chloroform–ethanol, and the solvent was evaporated off. The residue was dried *in vacuo* (phosphorus pentaoxide), and a portion of it (2 mg) was treated with 2M trifluoroacetic acid (1 mL) for 2.5 h at 105°. The solution was cooled and applied to a column (1 × 15 cm) of AG 1-X8 (OAc^-, 100–200 mesh) ion-exchange resin, and the column was eluted with water (15 mL), followed by 10mM acetic acid (10 mL). The combined eluates were lyophilized. The residue was acetylated with pyridine (0.5 mL) and acetic anhydride (0.3 mL), and the solution was evaporated. A solution of the residue in 9:1 (v/v) water–methanol (1 mL) was treated with sodium borohydride (10 mg) for 6 h at 4°. A further amount of sodium borohydride was added, and the solution was kept for 2 h at 22°. The remaining sodium borohydride was eliminated with 4M acetic acid, and the sodium ions were removed by treatment with AG 50W-X8 (H^+, 50–100 mesh) ion-exchange resin. After evaporation, boric acid was removed from the residue by repeated additions and evaporations of methanol (3 × 4 mL). The residue was treated with pyridine (1 mL) and acetic anhydride (0.65 mL) for 4 h at 22°. The resulting *O*-methylalditol acetates were estimated and identified by g.l.c.–m.s.

Mild acid-hydrolysis and remethylation of methylated oligosaccharide Fraction B-1. — The methylated oligosaccharide B-1 (5 mg) was treated with 50mM sulfuric acid for 75 min at 80°. The cooled solution was applied to a column (1 × 15 cm) of AG 1-X8 (OAc^-, 50–100 mesh) ion-exchange resin. The column was eluted with water (15 mL), and then with 5mM acetic acid (10 mL), and the combined eluates were lyophilized. The residue was methylated with iodomethane-d_3 (+99 atom%, Aldrich Chemical Co., Inc., Milwaukee, WI 53233) and methylsulfinylsodium, processed, hydrolyzed, and converted into the alditol acetates, which were identified and estimated by g.l.c.–m.s. (Table II), as just described.

Degradation by periodate oxidation–sodium borohydride reduction of oligosaccharide Fraction B-1. — Oligosaccharide Fraction B-1 (6 mg), in 0.1M sodium acetate (pH 4.5, 1.5 mL) containing 0.1M sodium metaperiodate, was kept in the dark for 16 h at 22°. The excess of sodium periodate was decomposed by the addition of 1,2-ethanediol (0.5 mL), and the salts were removed by gel filtration on a column

TABLE II

O-METHYL DERIVATIVES OBTAINED BY ACID HYDROLYSIS OF METHYLATED FRACTION B-1 OBTAINED FROM THE PREMENSTRUAL-PHASE GLYCOPROTEIN, BEFORE AND AFTER MILD ACID-TREATMENT, FOLLOWED BY DEUTERIOMETHYLATION

O-Methyl derivatives	*Molar ratio*	
	Before acid treatment[a]	*After acid treatment*
L-Fucose[b]		
2,3,4-	1.2	
D-Galactose[b]		
2,4-	0.6	
2,3,4-	Traces	9[d]
3,4,6-	0.6	
2,4,6-	Traces	
2,3,4,6-	0.6	1[d]
2-Acetamido-2-deoxy-D-glucose[c]		
3,6-	1.00	
2-Acetamido-2-deoxy-D-galactose[c]		
3,4,6-	1.00	
2-Acetamido-2-deoxy-D-galactitol[c]		
1,4,5-	0.4	
1,3,4,5-	0.8	
N-Acetylneuraminic acid[e]	1.0	0.3[f]

[a]Molar ratios relative to 2-acetamido-1,4,5-tri-*O*-acetyl-2-deoxy-3,6-di-*O*-methyl-D-glucitol. [b]Determined as alditol acetates and molar ratios calculated by g.l.c.[14]. [c]Determined as alditol acetates and molar ratio calculated by monitoring[15] *m/e* 158 or 116, and *m/e* 130 or 88. [d]Molar ratio of 3-*O*-[$^2H_3$]methyl-2,4,6-tri-*O*-methyl- to 3-*O*-[$^2H_3$]methyl-2,4-di-*O*-methyl-D-galactose. [e]Determined as methyl ester methyl glycosides by g.l.c. [f]Molar ratio relative to permethylated *N*-acetylneuraminic acid before acid treatment, and determined as in footnote *e*.

of Bio-Gel P-2. Carbohydrate-containing fractions were combined and lyophilized. A water solution of the residue was treated with sodium borohydride (8 mg) for 8 h at 4°, the remaining borohydride was eliminated by addition of 4M acetic acid, and the pH of the solution was adjusted to 5.4. The solution was applied to a column (0.8 × 12 cm) of AG 50W-X8 (H^+, 100–200 mesh) ion-exchange resin. The column was eluted with 5mM acetic acid (50 mL), the eluate was lyophilized, and the boric acid was removed from the residues by repeated additions and evaporations of methanol. The residue was treated with 0.5M hydrochloric acid (0.5 mL) for 8 h at 22°. The solution was applied to a column (0.8 × 16 cm) of AG 1-X8 (OAc^-, 200–400 mesh) ion-exchange resin. The column was eluted with water (20 mL) and the eluate lyophilized to give the periodate-oxidized oligosaccharide (for composition, see Table I).

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