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CHARACTERIZATION AND STRUCTURE OF A SIALIC ACID-CONTAINING HEXASACCHARIDE ISOLATED FROM HUMAN PREGNANCY URINE*

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ABSTRACT

A hexasaccharide containing sialic acid has been isolated from the urine of pregnant women. It contains D-galactose, D-glucose, *N*-acetyl-D-glucosamine, and sialic acid in the proportions of 2:1:1:2. Its structure, established by methylation analysis, enzymic digestion, and potassium borohydride reduction, is that of lacto-*N*-tetraose with one of the residues of sialic acid linked α-(2→3) to the terminal nonreducing residue of D-galactose, and the other sialic acid residue linked α-(2→6) to the *N*-acetyl-D-glucosamine residue. This hexasaccharide is related to human pregnancy, as it has not been found in nonpregnant, normal-woman urine and is present in human milk.

INTRODUCTION

Pregnancy is known to enhance the elimination of the bulk of nondialyzable carbohydrate components[1] and, in a previous work[2], we have shown that the urinary excretion of oligosaccharides is enhanced (200%) as early as the 17th week of pregnancy. This enhancement may be related either to the increase of oligosaccharides that are also present in normal urine or to the urinary excretion of new oligosaccharides. Six oligosaccharides containing L-fucose were isolated from the urine of pregnant women and characterized by Date[3,4] who has shown that they were absent in the urine of women who were neither pregnant nor lactating. These oligosaccharides were found to be identical with those isolated from human milk[5,6].

Maury[7] was the first to isolate oligosaccharides containing sialic acid from the urine of pregnant rat, showing that neuraminyllactose and neuraminyllactosamine excretion increases 2–3 fold during the course of pregnancy. Both oligosaccharides are constituents of normal urine. In the present work, undertaken to isolate sialic acid-

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containing oligosaccharides strictly related to pregnancy, a new hexasaccharide isolated from human pregnancy urine was characterized and its complete structure established. A similar hexasaccharide has been isolated from human milk[8].

RESULTS AND DISCUSSION

The hexasaccharide was obtained from the urine of pregnant women during the last trimester, as described before[9]. It was homogeneous in paper chromatography in three solvent systems, and in high-voltage paper electrophoresis at two pHs, as detected by the aniline oxalate reagent and the Schiff's reagent after periodate oxidation. It contained D-galactose, D-glucose, *N*-acetyl-D-glucosamine, and *N*-acetylneuraminic acid in the proportions of 2:1:1:2, as shown by several methods, and no amino acids (see Table I). After potassium borohydride reduction, no D-glucose was observed (0.07 residue) but D-glucitol was present (1.05 residue, see Table I). No change was observed for the other monosaccharides, indicating a D-glucose residue as reducing end.

In order to determine the sequence of the carbohydrate residues, both the hexasaccharide and the sialic acid-free derivative were submitted to enzymic hydrolysis with various glycosidases. None of the β-D-galactosidases or the *N*-acetyl-β-D-glucosaminidases were found to split off any D-galactose or *N*-acetyl-D-glucosamine residue from the hexasaccharide, indicating the presence of one sialic acid residue at the terminal, nonreducing end. The results obtained with the sialic acid-free oligosaccharide are summarized in Table II. It can be assumed that a D-galactose residue is located at the nonreducing end of the tetrasaccharide, as beef kidney *N*-acetyl-β-D-glucosaminidase did not liberate any *N*-acetyl-D-glucosamine before β-D-galactosidase digestion. This terminal D-galactose residue was poorly split off by jack-bean β-D-galactosidase (0.09 residue), whereas beef-liver β-D-galactosidase was more efficient (0.39 residue). Jack-bean β-D-galactosidase has been reported to liberate D-galactose very slowly from 2-acetamido-2-deoxy-3-*O*-β-D-galactopyranosyl-D-glucose[10,11], suggesting that the external β-D-galactopyranosyl group is β-(1→3)-linked to an *N*-acetyl-D-glucosamine residue; this structure was corroborated by the methylation analysis. After partial removal of D-galactose (0,39 mol/mol of saccharide) and of *N*-acetyl-D-glucosamine (0.35 mol/mol of saccharide), the disaccharide produced was identified as lactose by g.l.c. ($R_{Mannitol}$ 2.99 and 3.55). D-Galactose, *N*-acetyl-D-glucosamine, and lactose were produced by enzymic digestion in the molar proportions of 0.4:0.35:0.39, as determined by g.l.c., suggesting the structure:

α-NeuAc*p*-(2→ ?)-β-D-Gal*p*-(1→3)-β-D-GlcNAc-(1→ ?)-β-D-Gal-(1→4)-D-Glc

The final structure was established by methylation analysis (see Table III). The absence of 2,3,4,6-tetra-*O*-methyl-D-galactose from the hydrolyzate of the permethylated hexasaccharide confirmed the structure established by enzymic hydrolysis: an *N*-acetyl-α-neuraminosyl group at the nonreducing end is linked (2→3) to a D-galactose residue. The second *N*-acetyl-α-neuraminosyl group is linked (2→6)

TABLE I

COMPOSITION OF INTACT, SIALIC ACID-FREE, AND REDUCED HEXASACCHARIDE

Components	*Methods*[a]	*Hexasaccharide*			
		Intact		*Sialic acid-free*	*Reduced*
		%	*Molar ratios*[b]	*Molar ratios*[b]	*Molar ratios*[b]
Total neutral hexoses	Orcinol-H_2SO_4	36.6	3.05		
Gal	G.l.c. ($SiMe_4$)		2.06	2	1.94
	G.l.c. (F_3)		1.94	2.1	
Glc	G.l.c. ($SiMe_4$)		1.07	1.1	0.07[c]
	G.l.c. (F_3)		0.94	1.1	
Total hexosamines	Elson–Morgan	14.6	1		
GlcNAc	G.l.c. ($SiMe_4$)		1	1	1
GlcNAc	G.l.c. (F_3)		1	1	
$GlcNH_2$	Unichrom	15.8	1.08		
$GalNH_2$	Unichrom	0	0		
	G.l.c. ($SiMe_4$, F_3)	0	0		
N-Acetylneuraminic acid	Warren	47.9	2.3		
	Dische	48.5	2.4		
	G.l.c. ($SiMe_4$)		2.2		1.75
	G.l.c. (F_3)		2.31		
Amino acids	(Unichrom)				
Glu			0.07		
Gly			0.06		
Others			0.01		

[a]Abbreviations: $SiMe_4$, tetramethylsilyl; F_3, trifluoracetyl. [b]Molar ratios to *N*-acetyl-D-glucosamine (g.l.c.) or to D-glucosamine (colorimetric determinations). [c]With formation of D-glucitol (1.05 mole/mole of hexasaccharide).

TABLE II

RELEASE OF MONO- AND DI-SACCHARIDES BY VARIOUS GLYCOSIDASES FROM SIALIC ACID-FREE OLIGOSACCHARIDE

Compound released and method	*Glycosidase*			
	β-D-Galactosidase		*N-Acetyl-β-D-glucosaminidase*[a]	
	Jack bean[b]	*Beef liver*[c]	*Before*[d]	*After treatment with β-D-galactosidase*
D-Galactose[e]				
Colorimetric	0.09	0.40	0	
G.l.c.		0.39		
N-Acetyl-D-glucosamine[e]				
Colorimetric			0	0.35
G.l.c.				0.35
Lactose (g.l.c.)				0.39

[a]From beef kidney. [b]The release of D-galactose was estimated after a 24-h incubation at 37°. [c]After a 24-h incubation at 37° with β-D-galactosidase, the released D-galactose was estimated by a colorimetric assay. Subsequently, *N*-acetyl-β-D-glucosaminidase was added to the mixture which was kept for another 24 h at 37°. After that time, the release of *N*-acetyl-D-glucosamine was quantitatively determined by a colorimetric assay. The release of D-galactose, *N*-acetyl-D-glucosamine, and lactose was also determined by g.l.c. [d]The release of both D-galactose and *N*-acetyl-D-glucosamine was estimated after a 24-h incubation at 37°. [e]The release of monosaccharides is expressed in mol/mol of oligosaccharide.

TABLE III

IDENTIFICATION OF PARTIALLY METHYLATED DERIVATIVES OBTAINED FROM THE HYDROLYZATE OF PERMETHYLATED, INTACT, AND SIALIC ACID-FREE HEXASACCHARIDE BY G.L.C.

Methyl ethers	*Hexasaccharide*	
	Intact	*Sialic acid-free*
D-Galactose		
2,3,4,6-tetra[a]	−	+
2,4,6-tri[a]	+	+
D-Glucose		
2,3,6-tri[a]	+	+
2-Deoxy-2-(*N*-methylacetamido)-D-glucose		
4,6-di[b]	−	+
4-mono[b]	+	−

[a]Identified as methyl glycosides. [b]Identified as trimethylsilyl derivatives.

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to a 2-acetamido-2-deoxy-D-glucose residue, as shown by the production of the partially methylated derivatives of 2-amino-2-deoxy-D-glucose. The identification of the partially methylated monosaccharides showed that the 2-acetamido-2-deoxy-D-glucose residue is linked at C-3 with the external D-galactose residue, which is confirmed by the slow hydrolysis of this residue with jack-bean β-D-galactosidase. It also showed that the reducing D-glucose residue is bound to the internal D-galactose residue at C-4, as in lactose. All the data indicate that the hexasaccharide has the following structure:

α-NeuAc*p*-(2→3)-β-D-Gal*p*-(1→3)-[α-NeuNAc*p*-(2→6)]-β-D-GlcNAc*p*-(1→3)-β-D-Gal*p*-(1→4)-Glc

The origin of the urinary oligosaccharides is not fully elucidated. They are often thought to originate from the catabolism of serum or tissue glycoproteins, and of glycolipids[12]. The urinary hexasaccharide that is described in this paper does not seem to originate from serum-glycoprotein catabolism, as none of these glycoproteins is actually known to have such a carbohydrate moiety. Some glycolipids have carbohydrate chains with this type of structure, but we were unable to find a similar hexasaccharide in the urine of nonpregnant women[13].

An alternative hypothesis for the origin of urinary oligosaccharides is a biosynthetic pathway in which glycosyltransferases would add various monosaccharides to a D-glucose, D-galactose, or lactose molecule[14,15]. Pregnancy is known to greatly enhance the synthesis of lactose and, indeed, all the oligosaccharides containing L-fucose and sialic acid that were isolated from the urine of pregnant women contain a lactose residue at their reducing end. Moreover, a number of oligosaccharides containing L-fucose exhibit the lacto-*N*-tetraose structure[4] observed here.

In fact, all the oligosaccharides obtained from the urine of pregnant women are identical with the oligosaccharides isolated from human milk, but some of them are not related to pregnancy, neuraminyllactose for example. The urinary hexasaccharide that is described in this paper is the first urinary oligosaccharide containing sialic acid that can be related to pregnancy, as it has been observed in the urine of several pregnant women, whereas it was not found in normal human urine, and is identical with a hexasaccharide obtained from human milk[8]. It is probably synthetized in the mammary glands, though another origin (placental for example) cannot be completely ruled out.

EXPERIMENTAL

Materials. — Urine was collected from a healthy, nonsecretor pregnant woman during the third trimester of pregnancy, bacterial growth being prevented by the addition of Merthiolate (0.2‰). The hexasaccharide was obtained from the urine as previously described[9].

Analytical methods. — Neutral hexoses were determined by the orcinol–sulfuric acid method[16], and sialic acid by the thiobarbituric acid method[17] and by the diphenylamine method[18]. Hexosamines were determined with a Beckman Amino

Acid Analyzer Unichrom[19] and by a colorimetric determination[20] after hydrolysis for 4 h at 100° with 3M HCl. Neutral hexoses, *N*-acetylhexosamines, 6-deoxyhexoses, and sialic acid were also determined by g.l.c. as the trimethylsilyl[21] or trifluoroacetyl[22] derivatives of the methyl glycosides. Monosaccharide and disaccharide analysis was also performed by g.l.c. with mannitol as standard, after direct trimethylsilylation for 2 h, with a temperature programmed from 150° to 250° at a rise of 2°/min[23]. Under these conditions, a lactose standard gave two peaks ($R_{Mannitol}$ 2.98 and 3.35). Amino acid composition was determined with a Unichrom autoanalyzer after hydrolysis with 6M HCl for 7 h at 100° *in vacuo*. The solvent systems (all proportions v/v) for analytical paper chromatography were: (*A*) 5:5:1:3 pyridine–ethyl acetate–acetic acid–water, (*B*) 13:6 propanol–water, and (*C*) 2:1:2 ethyl acetate–pyridine–water. High-voltage, paper electrophoresis was performed at pH 1.9 in 1:3:36 formic acid–acetic acid–water, and at pH 6.4 in 25:1:1974 pyridine–acetic acid–water.

Removal of sialic acid. — As α-neuraminidase only liberated 65% of the *N*-acetylneuraminic acid residues, the quantitative removal of sialic acid from the hexasaccharide was performed by hydrolysis with 0.05M H_2SO_4 for 1 h at 80°. After dilution, the solution was filtered through a column of Dowex 1(X-4, HCO_3^-) ion-exchange resin suspended in water. The material eluted by water was freeze-dried and the residue submitted to preparative paper chromatography in solvent *A* for three days. Lateral strips were stained with aniline oxalate, the material eluted by water was freeze-dried, and the residue submitted to carbohydrate analysis (g.l.c.). All the sialic acid was split off and no loss of either neutral hexoses or *N*-acetylhexosamine was observed.

Reduction. — The hexasaccharide (2 mg) was reduced with potassium borohydride (2 mg) in water (0.5 ml) for 15 h at room temperature. The excess of borohydride was destroyed by addition of acetic acid, and the mixture was filtered through a column of Dowex 50 (X-8, 100–200 mesh, H^+) ion-exchange resin. The effluent and the washing solution (water) were concentrated *in vacuo*, and boric acid was removed as methyl borate. After the addition of mannitol, the reduced saccharide was analyzed by g.l.c. after methanolysis[21].

Enzymic degradation. — α-Neuraminidase (EC 3.2.1.18) from *Clostridium perfringens* (Grade V) was obtained from Sigma Chemical Co. (St. Louis, Mo., 63178). β-D-Galactosidase (EC 3.2.1.23) of jack bean was prepared according to Li and Li[10]. β-D-Galactosidase (IC 3.2.1.23) of beef liver was obtained from Sigma Chemical Co. (Lot no. 11.C. 7670, 0.32 unit/mg); it had very weak α-D-galactosidase activity (1 munit/mg) and *N*-acetyl-β-D-glucosaminidase activity (0.032 unit/mg), as tested with the *p*-nitrophenyl substrates. It did not hydrolyze lactose. Native and sialic acid-free oligosaccharides (0.6 μmol) were incubated with 0.18 unit of jack-bean β-D-galactosidase in 0.05M NaCl–0.01M phosphate buffer, at pH 7.2, for 24 h at 37° under toluene. The release of D-galactose was determined enzymically[24]. *N*-Acetyl-β-D-glucosaminidase (EC 3.2.1.30) from beef kidney was obtained from Boehringer Mannheim France SA (75001 Paris, Lot no. 7364103, 4 unit/ml); it showed neither

N-acetyl-α-D-glucosaminidase nor β-D-galactosidase activity in 0.15M NaCl–0.01M phosphate buffer at pH 7.2. One unit of enzyme activity (β-D-galactosidase and *N*-acetyl-β-D-glucosaminidase) is the amount of enzyme that liberates, in 1 min at 37°, 1 μmol of *p*-nitrophenol from the *p*-nitrophenyl glycosides used as the substrates.

The sialic acid-free oligosaccharide (0.6 μmol) was incubated with 0.12 unit of *N*-acetyl-β-D-glucosaminidase before and after incubation with the β-D-galactosidase of beef liver, and the mixture was kept for 24 h at 37°. The release of *N*-acetyl-D-glucosamine was determined as described by Reissig *et al.*[25]. After the incubation with β-D-galactosidase (beef liver) and *N*-acetyl-β-D-glucosaminidase (beef kidney), the reaction mixture was heated at 100° for 5 min, and the salts were removed by passage through columns of Dowex 50 (X-4, H^+) and Dowex 1 (X-4, HCO_2^-) ion-exchange resins suspended in water. The effluent and the washing solution were freeze-dried, and the residue was analyzed by g.l.c. after direct trimethylsilylation[23].

Methylation procedure. — The hexasaccharide was permethylated as its potassium salt[26]. The permethylation of both sialic acid-containing and sialic acid-free oligosaccharides (2 μmol) was performed in two steps according to Kuhn *et al.*[5] and to Hakomori[27]. The permethylation was monitored by t.l.c. (Silica gel G) of the methylated saccharides in 9:1 (v/v) chloroform–methanol. After hydroysis with 4M HCl for 4 h at 100°, the mixture was dried and submitted to preparative paper electrophoresis (Whatman paper 3 MM, pH 3.9, 3:10:487, v/v, pyridine–acetic acid–water, 7 V/cm for 4 h). D-Mannose and D-glucosamine hydrochloride were used as standards. After staining with ninhydrin and aniline oxalate, the "basic" and the "neutral" fractions were revealed and eluted with water. The methyl ethers were identified according to Fournet *et al.*[28]. The methyl ethers of D-glucosamine eluted from the "basic" fraction were analyzed by g.l.c. of their trimethylsilyl derivatives. The methyl ethers of the neutral monosaccharides obtained from the "neutral" fraction were identified as their methyl glycosides before and after acetylation.

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