ISOLATION AND CHARACTERIZATION OF MONOFUCOSYL MONOSIALYL DERIVATIVE OF LACTO N-NEOHEXAOSE OBTAINED FROM HUMAN PREGNANCY URINE

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SUMMARY : An octasaccharide containing fucose and sialic acid has been isolated from human pregnancy urine. It contains D-galactose, D-glucose, L-fucose, N-acetyl-D-glucosamine and sialic acid in the proportions of 3 : 1 : 1 : 2 : 1. Its structure is partially established by methylation analysis, enzymic digestion, potassium borohydride reduction and immunological studies. It derives from lacto-N-neohexaose. This octasaccharide is related to pregnancy : it has not been found in non pregnant, normal-women urine and the presence of similar strucutre in milk has been reported recently.

INTRODUCTION

Pregnancy is known to largely increase the urinary excretion of the bulk of nondialyzable glycoconjugates (1). In a previous work (2) we have shown that this increase was observed for the oligosaccharide fraction as early as the 17th week of pregnancy and similar results were observed by HALLGREN et al. (3). A number of fucooligosaccharides, absent from normal urine and so related to pregnancy, have been isolated and their structure established (3 - 6). Their presence in individual samples and their structures vary with the ABO or Lewis blood type of the donor. Disialyllacto-N-Tetraose has also been isolated from pregnancy urine (7) and its structure established (8). All these saccharides derived from lacto-N-tetraose or lacto-N-neotetraose and they were identical to milk saccharides (9 - 11).

The present work was then undertaken to investigate the presence in pregnancy urine of higher saccharides, as shown in milk, and in order to work out their structures.

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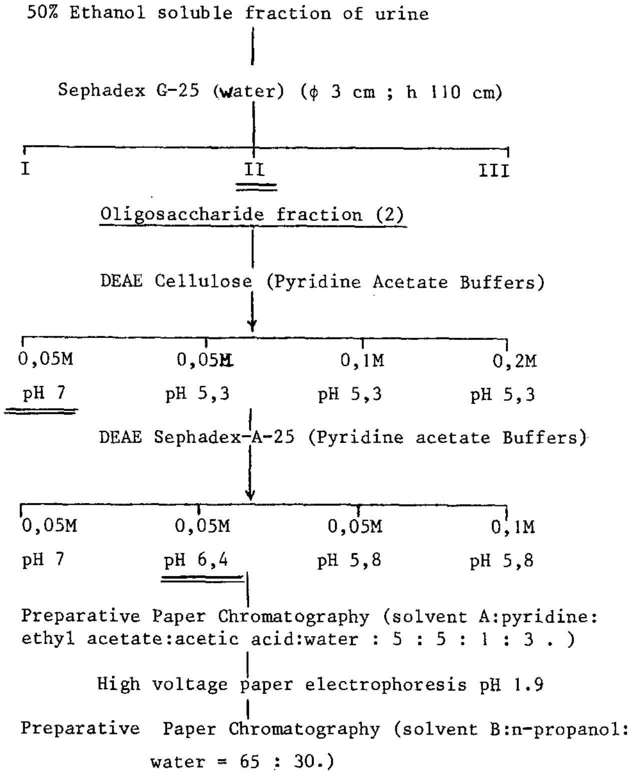


FIGURE I - Isolation of the octasaccharide .

MATERIALS AND METHODS

Preparation of the sialyloligosaccharide was carried out as described in Fig. 1. It was homogeneous in gel filtration and in paper chromatography (two solvents) and high voltage paper electrophoresis at pH 1.9 after staining with the anilin oxalate reagent and Schiff's reagent (following periodate oxidation).

Analytical techniques

Neutral hexoses, N-acetylhexosamines, 6-deoxyhexoses, and sialic acid were determined by gas-liquid chromatography (g.l.c.) as the trimethylsilyl (12) or trifluoroacetyl (13) derivatives of the O-methylglycosides. Aminoacid composition was determined with a Unichrom autoanalyzer after hydrolysis with 6M HCl for 7 h at 100°C in vacuo.

The solvent systems (all proportions v/v) for paper chromatography were (A) 5 : 5 : 1 : 3 pyridine-ethylacetate-acetic acid-water, (B) 65 : 30 n-propanol-water. High voltage paper electrophoresis was performed at pH 1.9 in 1 : 3 : 36 formic acid-acetic acid-water.

The native saccharide (2 mg) was reduced with potassium borohydride (20 mg) in water (1 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 (x8, 25-50 mesh, H^+ form) ion-exchange resin. The effluent and the washing solution (water) were concentrated in vacuo and boric acid was removed as methyl borate. After addition of internal standard, the reduced saccharide was analyzed by g.l.c. after methanolysis.

β-D-galactosidase (E C 3.2.1. 23) of Jack Bean meal was prepared in the laboratory according to Li and Li (14). β-D-galactosidase (E C 3.2.1. 23) of beef liver was obtained from Sigma (Lot n° 11 C 7670,00,32 unit/mg) ; it had very weak α-D-galactosidase activity (1 m unit/mg) and N-acetyl-β-D-glucosaminidase activity (0,032 unit/mg) as tested with the p-nitrophenyl substrates. It did not hydrolyse lactose. Only native oligosaccharide (1 µM) was incubated with either Jack Bean β-D-galactosidase 0.18 unit 0.05 M citrate buffer pH 4.6) or beef liver β-D-Galactosidase (0.18 unit 0.15 M NaCl - 0.1 M phosphate buffer pH 7.2) for 24 h at 37°C under toluene. The release of D-galactose was determined enzymically (15). N-acetyl-β-D-glucosaminidase (E C 3. 2. 1. 30) from beef kidney was otained from Boehringer Manhheim (France S.A. 75001 PARIS)(Lot n° 7364103, 4 units/ml) ; it showed neither N-acetyl-α-D-glucosaminidase nor β-D-galactosidase activities in 0.15 M NaCl - 0,01 M phosphate buffer at pH 7.2. One unit of enzyme activity is the amount of enzyme that liberates in 1 minute at 37°C 1 µmole of p-nitrophenol from the p-nitrophenylglycosides used as the substrates. Native oligosaccharide was incubated with 0.12 unit of N-acetyl-β-D-glucosaminidase before and after incubation with either β-D-galactosidases. The release of N-acetyl-D-glucosamine was determined as described by REISSIG et al. (16).

Permethylation of the native saccharide was performed according to HAKOMORI's (17) modified procedure. Potassium salt of reduced octasaccharide (1 mg) was sonicated in anhydrous dimethylsulfoxyde (1 ml) until complete dissolution. Methylsulfinylcarbanion (0,5 ml) was then added and the mixture sonicated for another 30 min under N_2. After cooling, methyl iodide (1 ml) was added slowly, then the mixture was sonicated for 30 min. The permethylation was monitored by thin layer chromatography (Silicagel G) of the methylated saccharide in 9 : 1 (v/v) chloroform-methanol. Methyl ethers were identified according to FOURNET et al. (22) after hydrolysis with 4 M HCl for 4 h at 100°C and resolution into "basic" and "neutral" fraction.

The hemagglutination inhibition assays were performed according to WEGMAN ant SMITHIES (19) with anti Le^a serum and appropriate red cells (Centre National de la Transfusion Sanguine - Paris).

RESULTS AND DISCUSSION

The composition of the octasaccharide is given in Table 1. It contains D-galactose, D-glucose, L-fucose, N-acetyl-D-glucosamine and N-acetyl neuraminic acid in the proportions of 3 : 1 : 1 : 2 : 1 and no aminoacids (Table 1). After potassium borohydride reduction, no D-glucose was observed (0.09 residue) but D-glucitol was present (0.85 residue, see Table 1). No change was observed for the other monosaccharides, indicating a D-glucose residue as reducing end.

TABLE I - Molecular[a] composition of native and reduced octasaccharide.

CONSTITUENTS	Native TMS derivatives	Reduced TMS derivatives	Reduced TFA derivatives
Fucose	1.1	1.16	0.98
Galactose	3	3	3
Glucose	1.2	0.09[b]	0[b]
N-acetylglucosamine	2.1	1.99	2.1
AcNeu	0.93	1.4	

[a] Molar ratios to 1 galactose

[b] With production of sorbitol (TMS = 0,85 residue)
(TFA = 0,86 residue)

Methylation studies were performed to determine the position of glycosidic linkages and the arrangment of the carbohydrate core. The partially O-methylated monosaccharides obtained from the hydrolysate of the permethylated octasaccharide are given in Table 2.

Presence of 1, 2, 3, 5, 6 - penta-O-methyl-D-glucitol was consistent with the results of borohydride reduction and with the occurence of a glucose substituted on C_4 at the reducing end of all fuco- and sialyloligosaccharides isolated from milk (7-13, 9-11, 20-25) and pregnancy urine (3-8, 26). Occurence of 2,4 d di-O-methyl-D-galactose from the hydrolysate indicated a branched core, one chain bearing a galactose residue as a non reducing terminal (2, 3, 4, 6 - tetra-O-methyl-D-galactose) and the other a sialic acid or a fucose residue linked with C_3 of a galactose (2, 4, 6 - tri-O-methyl-D-galactose). Two different methyl ethers were identified for N-acetyl glucosamine. The presence of 6-mono-O-methyl-2-deoxy-2-(N-methylacetamido)-O-glucose was consistent with the presence of a third non reducing end whilst the 3, 6 di-O-methyl derivative corresponded to a N-acetyl-lactosamine residue.

As the same glycosyl transferases are responsible for the synthesis of carbohydrate chains of blood group substances, of

TABLE II - Identification and determination of partially methylated derivatives obtained from the hydrolyzate of permethylated native octasaccharide by gaz-liquid chromatography.

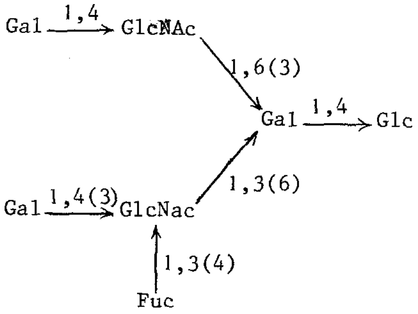
D-Glucose[a]		
1, 2, 3, 5, 6	penta-O-methyl sorbitol	1
D-Galactose[a]		
2, 3, 4, 6	tetra-O-methyl[b]	1.39
2, 4, 6	tri-O-methyl[b]	0.90
2, 4	di-O-methyl[b]	1
2-Deoxy-2-(N-methylacetamido)-D-glucose[c]		
3, 6	di-O-methyl	+
6	mono-O-methyl	+
L-Fucose[d]		
2, 3, 4	tri-O-methyl fucose [b]	1

[a] Identified as methyl glycosides and their acetylated derivatives.

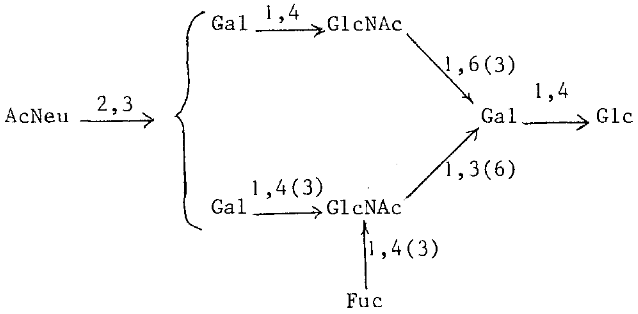
[b] Area ratios to 1, 2, 3, 5, 6 penta-O-methyl sorbitol.

[c] Identified as trimethylsilyl derivatives.

milk oligosaccharides (27) and likely of oligosaccharides isolated from urine (28-33), a structure is proposed for the core of the octasaccharide.



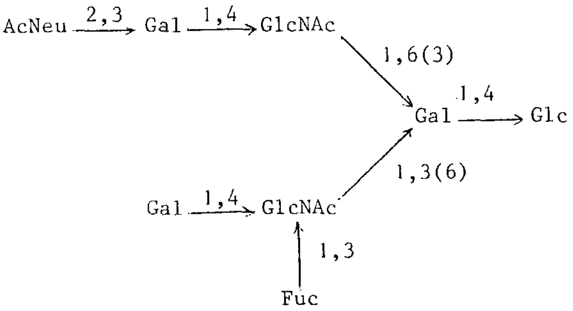
Fucose occurs in the oligosaccharides isolated from blood group substances anf from milk (24) in at least five linkages : α-1,2 and α-1,3 to galactose, α-1,3 and α-1,4 to N acetyl glucosamine and α-1,3 to glucose and sialic acid occurs in at least three different linkages: α-2,3 and α-2,6 to galactose and α-2,6 to N-acetyl glucosamine. Using only these linkages, there are theoretically 8 isomers :



Neither galactose nor glucosamine were released by any enzymic hydrolysis though the presence of a galactose as a non reducing end had been clearly worked out by methylation analysis. Similar results have been obtained by previous workers (34-35) for non reducing terminal galactose residues which were linked to N-acetyl glucosamine with a fucose on it. So it was assumed that sialic acid and fucose were located on different chains.

At last, serological studies showed that native octasaccharide had no Le^a activity, so that the Gal β-1,3 (Fuc α-1,4) GlcNAc structure was to be discarded.

From all these results, only two isomers are left in which the relative positions of the two chains and the anomeric configurations are given yet. These isomers have a carbohydrate core similar to the lacto-N-neohexaose core :



The presence of many isomers of monofucosyl mono-or disialyl-derivatives of lacto-N-neohexaose has been reported in milk recently (23-24). Only one has been obtained in an homogeneous state from pregnancy urine (this work) ; three others were identified as major compounds in mannose-containing (0.1, 0.3 residue to 1 glucose) urinary fractions (M. LEMONNIER : non published results). None of these octasaccharides was detected by paper chromatography in normal urine. Their presence in pregnancy urine, as soon as the 16th week of pregnancy, can be related to a milk-like secretion which occurs as early as the 12th week of pregnancy (6) and so ascertains the mammary origin of these urinary saccharides.

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