HYDRAZINOLYSIS AND NITROUS DEAMINATION OF GLYCOPROTEINS. EVIDENCE FOR A COMMON INNER CORE IN CARBOHYDRATE MOIETY.

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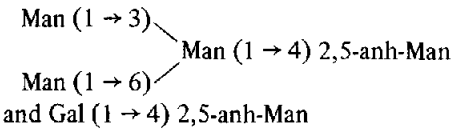
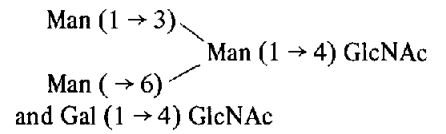
1. Introduction

Recent studies on glycoproteins indicate that the structure of the carbohydrate units of these proteins is very complex and some results suggest that it may be a carbohydrate common sequence in glycoproteins [1,2]. In an attempt to obtain further information about the chemical structure of these glycans, hydrazinolysis of glycoproteins and deamination with sodium nitrate of N-deacetylated glycans were applied to elucidate the inner core of the carbohydrate moiety. Nitrous deamination of N-deacetylated glycans specifically splits off glucosaminidic and galactosaminidic linkages [3–6] and yields oligosaccharides that contain 2,5-anhydro-hexose at their reducing end. This procedure was used on the following glycoproteins: α-acid glycoproteins, fetuin, lactotransferrin and transferrin.

In this communication, we put into evidence that the core of carbohydrate side chains of each glycoprotein we have studied, possesses the same inner sequence. For each glycoprotein, two oligosaccharides have been obtained with a 2,5-anhydro-D-mannose (2,5-anh-Man) at their reducing end. Methylation and other structural analysis lead us to propose the two undermentioned common structures:



that derived from the sequence



2. Materials and methods

2.1. *Glycoproteins.*

Fetuin was obtained from SIGMA. Human serotransferrin was isolated according to Roop and Putman [7]. Human α_1-acid glycoprotein was isolated from pooled normal human plasma according to Bürgi and Schmid [8]. Human lactotransferrin was prepared by resin exchange chromatography of the ammonium sulfate precipitate of human milk [9]. Asialoglycoproteins were obtained by partial hydrolysis (0.05 M HCl ; 100°C; 1h). The galactose/mannose ratio of each desialized glycoprotein was similar to that of the native glycoprotein.

2.2. *Hydrazinolysis and nitrous deamination.*

About 500 mg of each asialoglycoprotein, -fetuin, α_1-acid glycoprotein, transferrin and lactotransferrin-, were treated with 5 ml of hydrazine and kept in a sealed tube at 100°C for 30 hr. The reaction mixture

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was then evaporated under reduced pressure with 500 ml of toluol. The dry residue was dissolved in 0.5 ml of methanol and 7 ml of 15 p. 100 acetic acid, previously cooled at 4°C. The mixture was then passed through a Sephadex G-50 column (3 X 90 cm) to obtain N-deacetylated glycans (fig.1).

Nitrous deamination of N-deacetylated glycans was carried out as described by Horton et al. [10]. In a similar experiment, N-deacetylated glycans (100 mg) were dissolved in 6 ml of distilled water mixed with sodium nitrite (166.8 mg) and glacial acetic acid (0.90 ml). The mixture was kept at room temperature for 16 hours with occasional stirring, and then passed through a Dowex 50 X 8 ('mesh' 25–50; H^+) column followed by a Duolite A 102 D ('mesh' 25–50; HCO_3^-) column. A neutral fraction containing saccharides was obtained.

2.3. *Tritium labeling of oligosaccharides*

The molar ratio of the saccharides, obtained by nitrous acid deamination as previously described, was effected by a ^3H borohydride reduction. In a typical experiment, the resulting products of the nitrous acid deamination were dissolved in distilled water and sodium [^3H] borohydride (10 nmol; specific activity 10 Ci/mM) was added. The mixture was stirred for 2 hr at room temperature, after which an excess of unlabeled sodium borohydride was added and the mixture kept for 2 hr. The excess of borohydride was destroyed with few drops of glacial acetic acid and the labeled saccharides desalted on paper electrophoresis at pH 3.9 in pyridine–ethyl acetate–water (15:30:1935) buffer. The purified saccharides were chromatographed in the solvent: pyridine-ethyl acetate-acetic acid-water (5:5:1:3) and the sheet of Whatman No 3 paper was cut out every centimeter and counted for the radioactivity.

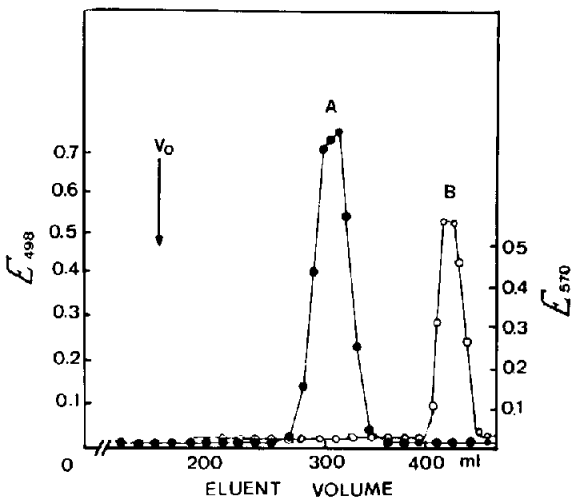


Fig.1. Gel filtration through a Sephadex G-50 column 3 X 90 cm) of α_1-acid glycoprotein treated by hydrazin. Fractions of 4.5 ml in volume were collected. Samples (0.1 ml) of each fraction were analyzed by phenol sulfuric acid method, measured at 498 nm (–●–●–) and the ninhydrin procedure without hydrolysis, at 570 nm (–○–○–). The vertical line indicates the position of the void volume (Vo). Fraction A contained N-deacetylated glycans.

2.4. *Isolation and structure of oligosaccharides*

Oligosaccharides that derived from the nitrous acid deamination of these glycoproteins were analyzed and isolated by preparative paper chromatography in the solvent 1: pyridine–ethyl acetate–acetic acid–water (5:5:1:3) and revealed with the urea–chlorohydric acid reagent [11]. Oligosaccharide structure determination was carried out as follows: oligosaccharides were reduced as previously described and methanolyzed with 1.5 M chlorohydric acid in anhydrous methanol at 80°C for 24 hr in a sealed tube for their molar ratio in monosaccharides. Separation of methylglycosides and polyols was achieved on gas-liquid chromatography as their trimethyl ether derivatives (fig.2) [12]. A Perkin-Elmer F_{11} instrument with flame ionization detector was used and separation was carried on a glass column packed with 3 p. 100 OV-17 on chromosorb W AW HMDS. Nitrogen was used as the carrier gas with a flow rate of 15 ml per min. The temperature indicated 110°C for 15 min then a temperature gradient of 1°C/min from 110°C to 180°C was applied. Erythritol was used as an internal standard.

Two permethylation cycles of reduced oligosaccharides were carried out as described by Hakomori et al. [13]. Methylated oligosaccharides were methanolyzed and analyzed by gas-liquid chromatography as follows: stainless steel column was filled with 3 p. 100 Carbowax 6000 on chromosorb W AW HMDS. Nitrogen was used as the carrier gas with a flow rate of 15 ml per minute. The column temperature was 160°C.

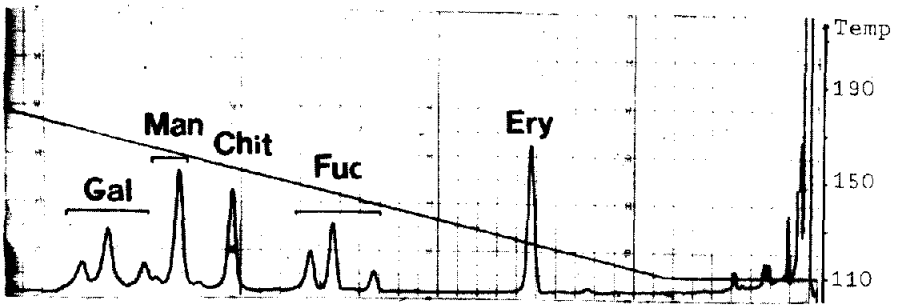


Fig. 2. Neutral oligosaccharides commonly occuring in deaminated glycopeptides were separated and analyzed on gas-liquid chromatography under their derivative form: *O*-trimethylsilyl ethers of the methyl glycosides. Chromatography was carried out on a glass column (0.3 × 300 cm) packed with 3 p. 100 OV 17 on chromosorb W AW HMDS. Nitrogen was used as the carrier gas with a flow rate of 15 ml per min. The temperature column indicated 110°C for 15 min then a temperature gradient of 1°C/min from 110 to 180°C was applied. *Abbreviations:* Gal: D-galactose; Man: D-mannose; Chit: chititol or 2,5-anhydro-D-mannitol; Fuc: L-fucose, Ery: Erythritol

3. Results and discussion

Paper chromatography analysis of nitrous deaminated products obtained from the different asialoglycoproteins, -lactotransferrin, α_1-acid glycoprotein, fetuin, transferrin-, shows (fig.3) that they all contain 2,5-anhydro-D-mannose (compound 3; R_{Gal} 0.98) and two neutral oligosaccharides (compound 1; R_{Gal} 0.27 and compound 2; R_{Gal} 0.61). The molar ratio of these oligosaccharides established by a [^{3}H] borohydride reduction is resumed in the table and illustrated by the fig.4.

The lactotransferrin diagram contains an additional peak which corresponds to the reduced oligosaccharide 2 bis which was not previously detected by paper chromatography analysis as its unreduced form.

Saccharides 1, 2 and 3 that belong from each glycoprotein were separately studied for their composition and sequence:

Compound 1. Whatever its origin may be, this oligosaccharide contains D-mannose and 2,5-anhydro-D-mannose in molar ratio 3/1; the latter disappears after borohydride reduction while appears a 2,5-anhydro-D-mannitol. Methanolysis of this reduced and methylated tetrasaccharide leads to the 1,3,6 tri-*O*-methyl–2,5-anhydro-D-mannitol, 2,3,4,6 tetra-*O*-methyl-D-mannoside and 2,4 di-*O*-methyl-D-mannoside. The sequence of this common oligosaccharide is as follows:

Man (1 → 3)
Man (1 → 4) 2,5-anh-Man
Man (1 → 6)

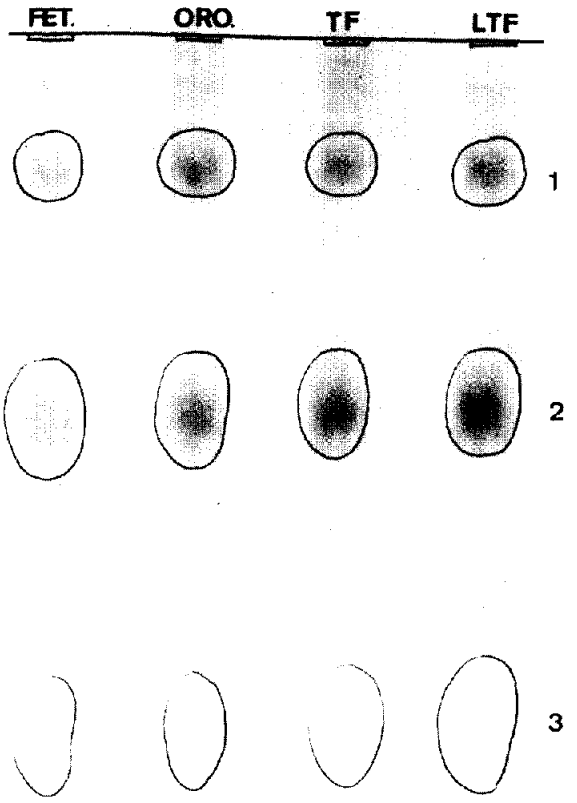


Fig. 3. Paper chromatography of deaminated products of N-deacetylated glycans. Paper chromatography was carried out on Whatman No 3 by the descending technique, using solvent 1 for 20 h at room temperature. Staining reagent: urea–chlorohydric acid. *Abbreviations:* FET: fetuin; ORO: orosomucoid; TF: transferrin; LTF: lactotransferrin. Compounds 1 and 2 are oligosaccharides, compound 3: 2,5-anhydro-D-mannose.

Table 1
Molar ratio of ^{3}H-reduced saccharides obtained by hydrazinolysis-nitrous acid deamination of asiaglycoproteins

	Compound 1	Compound 2	Compound 2 bis	Compound 3
R_{Gal}	0.27	0.61	0.61	0.98
Asialoglycoproteins				
α_1-acid glycoprotein	1.38	4.07	0	1.0
Transferrin	1.0	2.1	0	1.0
Lactotransferrin	1.0	1.5	0.8	0.20
Fetuin	1.15	3.87	0	1.0

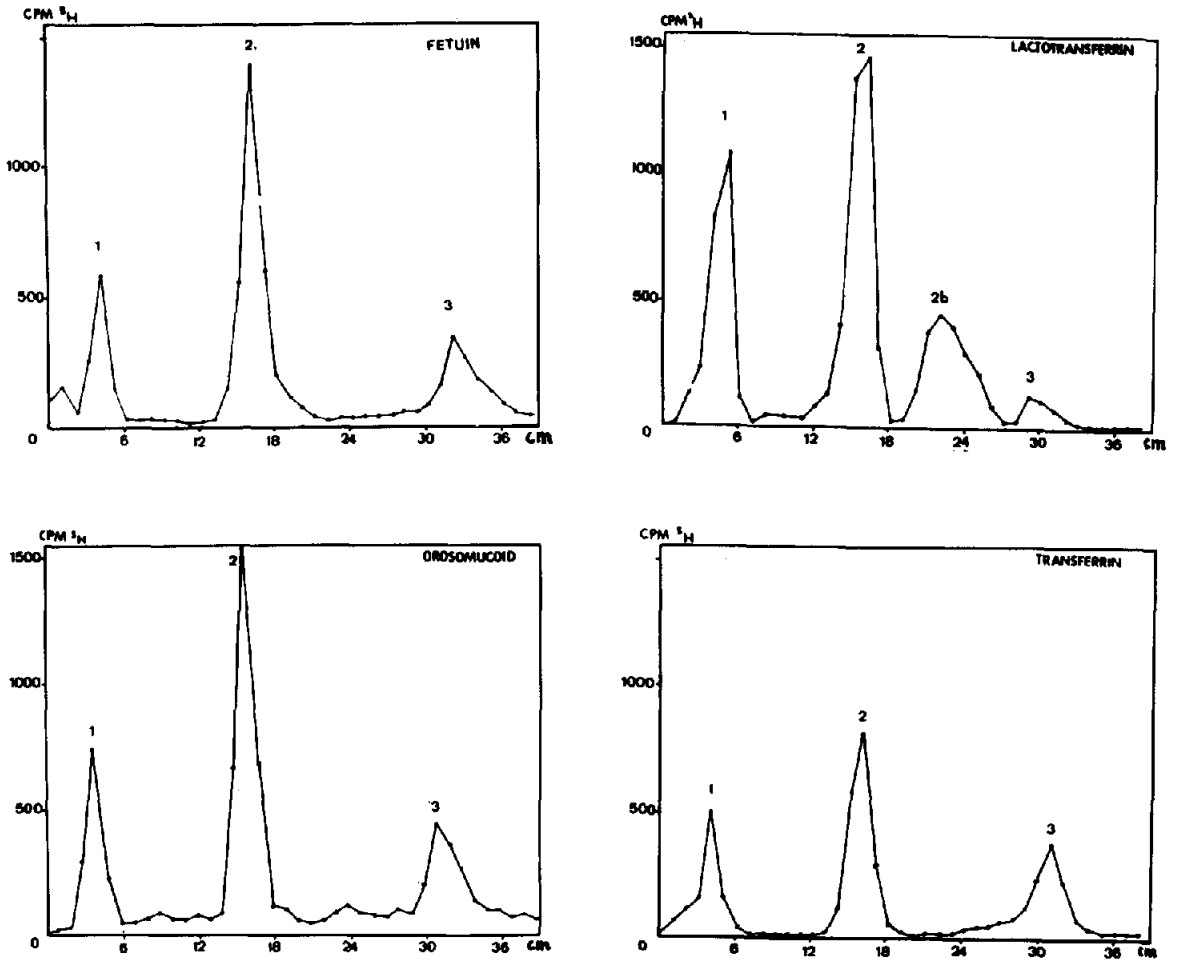
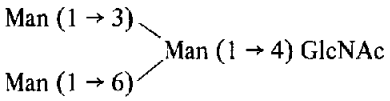


Fig.4. Paper chromatography of ^{3}H-reduced saccharides that derived from nitrous deamination products of asialoglycoproteins: orosomucoid, fetuin, transferrin, lactotransferrin. Paper chromatogram was cut out every 1.0 cm and counted for the radioactivity The molar ratio of the saccharides 1,2,2 bis and 3 are expressed in table 1.

This tetrasaccharide structure can therefore be tentatively assigned to the core of the glycans and belongs to the sequence:

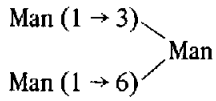


This structure is quite different from that proposed by Jamieson et al. [14] for the human serotransferrin and Sato et al. [15] for the human α_1-acid glycoprotein. But on the other hand, it agrees with the α_1-acid glycoprotein structure described by Hatcher and Jeanloz [16].

Compound 2 is formed by D-galactose and 2,5-anhydro-D-mannose in the ratio 1/1, this last residue being at the reducing end. Methanolysis of the reduced and methylated oligosaccharide leads to the 1,3,6 tri-*O*-methyl 2,5-anhydro-D-mannitol and 2,3,4,6 tetra-*O*-methyl-D-galactoside. The partial structure of this disaccharide is Gal (1 → 4) 2,5-anh-Man and derives from a Gal (1 → 4) GlcNAc sequence of the polysaccharide chain. This compound so called N-acetyllactosamine has been found in partial hydrolysate of orosomucoid [17] and fetuin [18] and belongs from the external part of the glycans.

Compound 2 bis, being only present in lactotransferrin is formed by D-galactose and 2,5-anhydro-D-mannitol in the molar ratio 1/1. The latter is at the reducing end. This disaccharide differs from the last one in the galactosyl linkage.

Compound 3. Its rate factor (R_{Gal} 0.98) is identical with that of the standard 2,5-anhydro-D-mannose. After reduction with an excess of sodium borohydride the reduced compound is analyzed on gas-liquid chromatography as the trimethylsilylated ether derivatives. Only one peak is obtained, having a similar relative retention time that the standard 2,5-anhydro-D-mannitol (R_t 0.48, relative to erythritol). The presence of free 2,5-anhydro-D-mannose demonstrates that the sequence GlcNAc → GlcNAc is present in both glycans. Moreover it has been shown that this free 2,5-anhydro-D-mannose derived from the *N*-acetylglucosamine residue linked to the peptide chain [19]. The occurence of three D-mannose residues having the precise sequence



in position immediately adjacent to the di-*N*-acetylchitobiose linked glycosidically to the amide group of asparagine has also been reported in a variety of proteins including Ige [20,21], Igm [22], IgG immunoglobulins [23], and some oligosaccharides accumulated in the liver of GM_1-gangliosidosis, type I [24]. However the occurence of this core structure of carbohydrate unit may not be universal features.

Acknowledgements

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