Carbohydrate Research, 46 (1976) 75–86

HYDRAZINOLYSIS AND NITROUS ACID DEAMINATION OF THE CARBOHYDRATE MOIETY OF α_1-ACID GLYCOPROTEIN

BERNARD BAYARD AND BERNARD FOURNET

Institut de Recherches sur le Cancer de Lille (Institut Jules Driessens) et U 124 de l'INSERM, B.P. 3567, 59020 Lille (France).
Laboratoire de Chimie Biologique de l'Université des Sciences et Techniques et L.A. au C.N.R.S. n° 217, B.P. n° 36, 59650 Villeneuve d'Ascq (France)

(Received March 4th, 1975; accepted for publication in revised form, May 30th, 1975)

ABSTRACT

Hydrazinolysis followed by nitrous acid deamination of α_1-acid glycoprotein gave acidic and neutral mono- and oligo-saccharides that contain 2,5-anhydro-D-mannose as reducing residue: α-D-Man*p*-(1→3)-[α-D-Man*p*-(1→6)]-β-D-Man*p*-(1→4)-2,5-anhydro-D-mannose (**1**), β-D-Gal*p*-(1→4)-2,5-anhydro-D-mannose (**3**), 2,5-anhydro-D-mannose, and two *N*-acetylneuraminic acid-containing oligosaccharides having the common partial sequence: NeuNAc-(2→?)-[β-D-Gal*p*-(1→4)-2,5-anhydro-D-mannose] (**5**). This specific cleavage of 2-amino-2-deoxy-D-glucosyl linkages released almost quantitatively a very limited number of saccharides. Reduction with sodium borotritide of the products of cleavage allowed the precise determination of the molar proportion of **1**, **3**, and free 2,5-anhydro-D-mannose.

INTRODUCTION

Structural investigations of the complex carbohydrate chains of α_1-acid glycoprotein based upon partial hydrolysis[1–4] and acetolysis[5] are rather tedious. Alkaline hydrolysis with sodium hydroxide, followed by nitrous acid deamination of α_1-acid glycoprotein gave, as fragmentation products, modified *N*-acetylneuraminic acid-containing oligosaccharides with a 2,5-anhydro-D-mannose reducing residue, but no information about structure of the "core" of the carbohydrate moiety was reported[6]. In the present work, selective cleavage of 2-acetamido-2-deoxy-D-glucosyl linkages with hydrazine (de-*N*-acetylation) and sodium nitrite (nitrous deamination) was applied to α_1-acid glycoprotein, in an attempt to obtain further information about the chemical structure of the "core" moiety.

Previously, the hydrazinolysis–nitrous deamination procedure has been used for the structure characterization of methyl glycosides[7], glycosaminoglycans[8–10] and acidic polysaccharides[11], although incomplete de-*N*-acetylation and partial destruction of monosaccharide residues or polysaccharide chains have been noted[10,12]. More recently, the selective and quantitative cleavage of the glucosaminyl linkage was

investigated[13–16], and this method was used for the structural identification of various glycoproteins[15] (fetuin, ovalbumin, ovomucoid, transferrin, lactotransferrin).

EXPERIMENTAL

Materials. — Crystalline 2,5-anhydro-D-mannitol was prepared by the method of Bera *et al.*[17], the methyl glycosides of D-mannose and D-galactose were prepared by methanolysis of the corresponding monosaccharides[18], and the di-, tri-, and tetramethyl ethers of methyl α,β-D-mannopyranoside by partial methylation of methyl α,β-D-mannopyranoside[19]. α_1-Acid glycoprotein was isolated from pooled, normal human plasma[20]. Sialic acid-free α_1-acid glycoprotein was obtained by partial hydrolysis with 0.05M hydrogen chloride for 1 h at 100°. The D-galactose to D-mannose ratio (1:1.14) of the sialic acid-free glycoprotein was similar to that of the native glycoprotein (Table I).

TABLE I

COMPOSITION OF THE CARBOHYDRATE MOIETIES OF NATIVE AND SIALIC ACID-FREE α_1-ACID GLYCOPROTEIN

Monosaccharide component	*Native α_1-acid glycoprotein*	*Sialic acid-free α_1-acid glycoprotein*
N-Acetylneuraminic acid[a] (%)	10.03	0
Neutral monosaccharides[b] (%)	16.54	17.89
D-Mannose	7.4	8.1
D-Galactose	8.5	9.3
L-Fucose	0.6	0.4
Ratio Gal/Man[c]	1.14	1.14
N-Acetylglucosamine[d]	16.7	17.2
Total carbohydrate	43.27	35.09

[a]Determined by the diphenylamine acid method[24]. [b]Determined by the orcinol–sulfuric method[22]. [c]The molar ratio of D-mannose, D-galactose, and L-fucose was determined with the aniline phtalate method. [d]Determined by the Elson–Morgan procedure[23] after acid hydrolysis (4M hydrochloric acid, 4 h, 100°).

Analytical methods. — Paper chromatography was performed on Whatman No. 3 paper with solvent A: 5:5:1:3 (v/v) pyridine–ethyl acetate–acetic acid–water. Paper electrophoresis was conducted in 3:10:187 (v/v) pyridine–acetic acid–water at pH 3.9. Chromatograms and electrophoregrams were dried and revealed with the urea–hydrochloric acid reagent[21].

Separation of methyl glycosides and polyols was achieved by g.l.c. of the per-*O*-trimethylsilyl ethers[18] with a Perkin–Elmer F_{11} instrument equipped with a flame-ionization detector and a glass column (0.3 × 300 cm) packed with 3% OV-17 on Chromosorb W-AW-HMDS, with nitrogen as the carrier gas at a flow rate of 15 ml/min, and a column temperature of 110° for 15 min, then raising with a gradient

of 1°/min to 180°. Erythritol was the internal standard. The trimethylsilyl ethers were obtained by dissolving the dried sugar in 1:1:5 (v/v) hexamethyldisilazane–chlorotrimethylsilane–pyridine purchased from Sigma Chemical Co (Saint Louis, Mo. 63178, U.S.A.). Total hexoses were determined by the orcinol–sulfuric acid procedure[22], hexosamines by the Elson–Morgan reaction[23], and *N*-acetylneuraminic acid by the diphenylamine method[24].

Two permethylation cycles were applied to the reduced oligosaccharides, as described by Hakomori[25], and the permethylated oligosaccharides were treated with 1.5M methanolic hydrogen chloride (1 ml) for 24 h at 80° in a sealed tube. Analysis of the various methylated alditols and glycosides was achieved by g.l.c. with a Perkin–Elmer F_{11} instrument on a stainless-steel column packed with 3% Carbowax 6000 W-AW-HMDS, nitrogen being the carrier gas at a flow rate of 15 ml/min and the column temperature at 160°.

Hydrazinolysis. — α_1-Acid glycoprotein (100 mg) was treated with hydrazine (1 ml) as previously described[16]. The mixture was passed through a Sephadex G-50 column (3 cm × 90 cm) to give two peaks (Fig. 1):

Fraction A (80% by weight of the carbohydrate moiety of α_1-acid glycoprotein) was composed of D-galactose, D-mannose, and 2-amino-2-deoxy-D-glucose and contained no amino acids. Traces of 2-acetamido-2-deoxy-D-glucose (less than 2% in

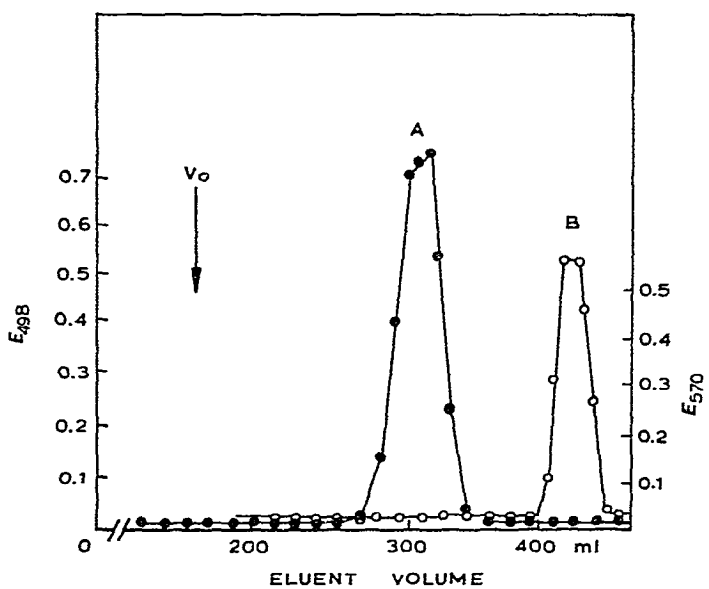


Fig. 1. Gel filtration through a Sephadex G-50 column (3 × 90 cm) of the hydrazine-treated α_1-acid glycoprotein. Fractions of 4.5 ml were collected and samples (0.1 ml) of each fraction were analyzed by the phenol–sulfuric acid method (measured at 498 nm) (—●—) and the ninhydrin procedure without hydrolysis (measured at 570 nm) (—○—). The vertical arrow indicates the position of the void volume (V_o). Fractions 62–71 were pooled and contained de-*N*-acetylated polysaccharide chains (Peak A).

term of total hexosamine) were estimated by g.l.c. after nitrous deamination and methanolysis. The galactose to mannose ratio (1:1.14) was similar to that of the native glycoprotein.

Fraction B was a mixture of amino acids, hydrazine, acethydrazide, and was not further studied.

Nitrous acid deamination. — The nitrous acid deamination of the purified de-*N*-acetylated glycans (Fraction A) was performed as described by Horton *et al.*[26]. The inorganic ions were removed by passing the reaction mixture through columns of

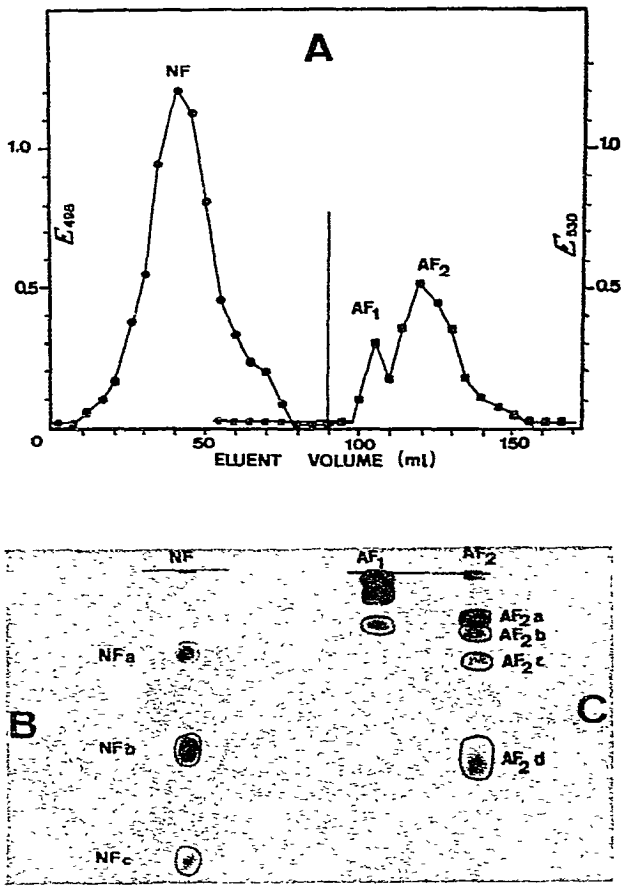
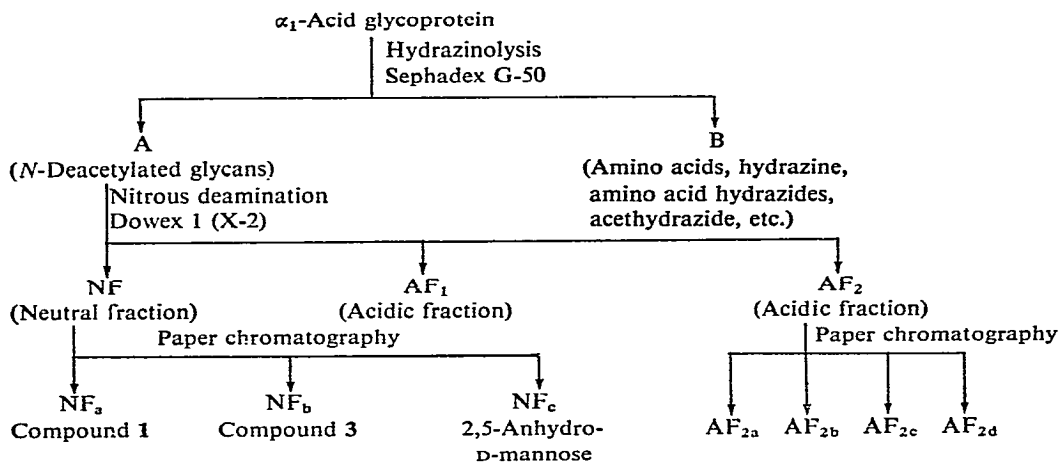


Fig. 2. *A.* Ion-exchange chromatography on a column (2 × 30 cm) of Dowex 1 (X-2) of the peak A (de-*N*-acetylated glycans) after deamination with nitrous acid. The column was first eluted with distilled water and then with 0.2% formic acid solution at the flow rate of 20 ml/h and 5-ml fractions were collected. Samples (0.1 ml) of each fraction were analyzed by the phenol–sulfuric acid method[20] (measured at 498 nm) (—●—) and by the diphenylamine procedure[23] (measured at 530 nm) (—■—). Fractions 6–12, 20–21, and 23–28 were collected to yield the neutral fraction NF and the two acidic fractions, AF_1 and AF_2, respectively. The vertical line indicates that the column is eluted with 2% formic acid.

B and *C:* Paper chromatography in solvent A of the fractions NF, AF_1, and AF_2 previously described in A. The paper chromatograms were stained with the urea–phosphoric acid reagent[25].

Dowex 50 (X-8, H^+, 25–50 mesh) and Duolite A-102-D (HCO_2^-, 25–50 mesh). The effluents and washings (500 ml) were combined and evaporated under reduced pressure. The residue was subsequently analyzed by paper chromatography (solvent A) and paper electrophoresis.

Isolation and purification of oligosaccharides. — Acidic and neutral saccharides having a 2,5-anhydro-D-mannose residue as reducing end, obtained by nitrous acid deamination, were separated by ion-exchange chromatography on a column (2 cm × 30 cm) of Dowex 1 (X-2, HCO_2^-, 200–400 mesh) (Fig. 2A). The neutral fraction (NF) (70% by weight), eluted from the resin with water, was analyzed and fractionated by paper chromatography in solvent A (Fig. 2B). D-Galactose, D-mannose, and 2,5-anhydro-D-mannose were present in the molar ratio 0.5:0.7:1.0. No peak corresponding to free hexitols, L-fucose, and 2-acetamido-2-deoxy-D-glucose were detected on the gas–liquid chromatogram. This fraction was resolved by paper chromatography in solvent A (Fig. 2B) into three major subfractions, NF_a (R_{Gal} 0.27), NF_b (R_{Gal} 0.61), and NF_c (R_{Gal} 0.98), in the respective yields of 25, 25, and 6% (by weight of the carbohydrate moiety of the α_1-acid glycoprotein) (Scheme 1).



Scheme 1. Hydrazinolysis and nitrous deamination of α_1-acid glycoprotein.

The acid fraction (AF), eluted from the column of Dowex 1 (X-2) resin with 2% formic acid, was resolved into two subfractions, AF_1 and AF_2 (Fig. 2A), in the respective yields of 2 and 6.3% (by weight of the carbohydrate moiety of α_1-acid glycoprotein). Subfraction AF_1 contained high-molecular-weight carbohydrate compounds due to the fact that incomplete de-*N*-acetylation of 2-acetamido-2-deoxy-D-glucopyranose residues with hydrazine prevents a specific cleavage of the 2-acetamido-2-deoxy-D-glucopyranosyl bonds by nitrous deamination. This fraction was not analyzed in detail. Subfraction AF_2 (Fig. 2C) contained diphenylamine-positive material, D-galactose, and 2,5-anhydro-D-mannose. No D-mannose and L-fucose were detected by g.l.c. This fraction was resolved by paper chromatography

into four bands: AF_{2a} (R_{Gal} 0.14), AF_{2b} (R_{Gal} 0.20), AF_{2c} (R_{Gal} 0.29), and AF_{2d} (R_{Gal} 0.61).

Tritium labelling of oligosaccharides. — The molar ratio of the three compounds, NF_a, NF_b, and NF_c, obtained by nitrous acid deamination of α_1-acid glycoprotein and sialic acid-free α_1-acid glycoprotein was determined by sodium borotritide reduction[16]. In a typical experiment, the compounds were dissolved in distilled water and sodium borotritide (10 nmol; specific activity 10 Ci/mmol) was added. The mixture was stirred for 2 h at room temperature, after which an excess of sodium borohydride was added and the mixture kept for 2 h. The excess of borohydride was destroyed with a few drops of glacial acetic acid and the labelled compounds desalted by paper electrophoresis at pH 3.9 in 1:2:129 (v/v) pyridine–ethyl acetate–water buffer. The labelled and purified compounds were chromatographed in solvent A and their radioactivity counted (Fig. 3). α_1-Acid glycoprotein and its sialic acid-free derivative gave different proportions of NF_a, NF_b, and NF_c: 0.99:1.99:1.00 and 1.38:4.07:1.00, respectively.

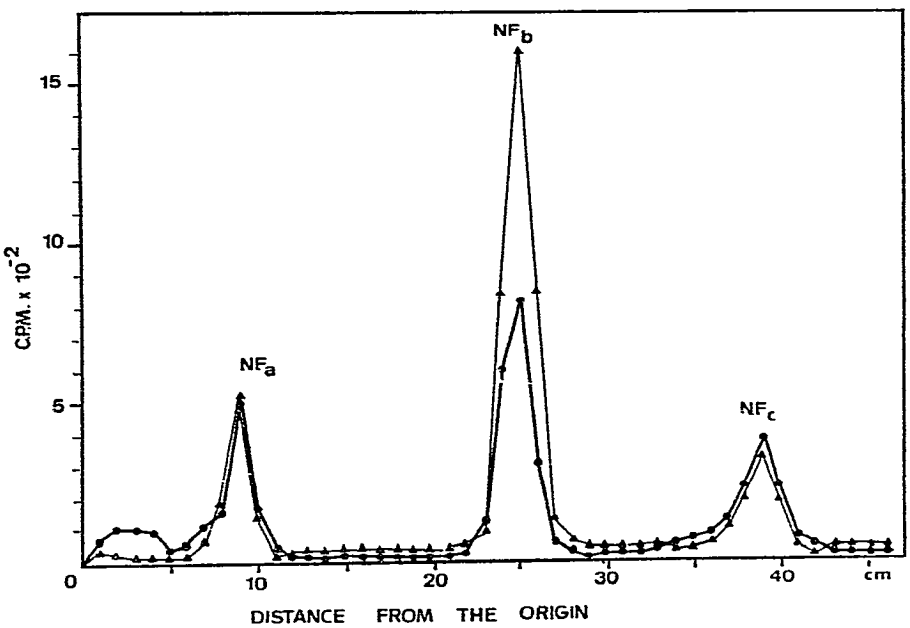


Fig. 3. Paper chromatography of the neutral fraction (NF), labelled by reduction with sodium borotritide, derived from native (—●—●—●—) and sialic acid-free (—▲—▲—▲—) α_1-acid glycoprotein. The paper chromatogram was cut out every 1.0 cm and counted for radioactivity. Letters NF_a, NF_b, and NF_c indicate the reduced saccharides (see Scheme 1).

Composition and structure of the oligosaccharides isolated from the neutral fraction (NF). — On paper chromatography, fractions NF_a, NF_b, and NF_c appeared homogeneous in solvent A. After elution from the chromatogram, each compound was analyzed for its composition and structure (Table II):

TABLE II

CHEMICAL COMPOSITION OF THE NEUTRAL FRACTION (NF) OBTAINED FROM NATIVE AND SIALIC ACID-FREE α_1-ACID GLYCOPROTEIN BY HYDRAZINOLYSIS AND NITROUS DEAMINATION

Component	*Yield*[a] (%)	R_{Gal}[b]	*Molar ratio*[c] *of degraded products obtained from*		*Composition of the fractions (molar ratio)*[d]		
			Native α_1-acid glycoprotein	*Sialic acid-free α_1-acid glycoprotein*	D-*Galactose*	D-*Mannose*	*2,5-Anhydro*-D-*mannose*
Neutral fraction (NF)	70				0.5	0.7	1.0
Compound NF_a	25	0.27	0.99	1.38	0	3.2	1.0
Compound NF_b	25	0.61	1.99	4.07	1.1	0	1.0
Compound NF_c	6	0.98	1.00	1.00	0	0	+

[a]Yield of the fractions (NF_a, NF_b, and NF_c) obtained by hydrazinolysis and nitrous deamination of native α_1-acid glycoprotein. [b]In solvent A. [c]Molar ratio of the carbohydrate components obtained after borotritide reduction, expressed in mole per carbohydrate units. [d]The determination of these compounds was obtained by g.l.c. on a OV-17 column after borohydride reduction. The values are expressed in terms of the 2,5-anhydro-D-mannitol.

Compound NF_a. Analysis by g.l.c. after reduction and methanolysis indicated the presence of methyl D-mannoside and 2,5-anhydro-D-mannitol in the ratio 3.2:1.

Fraction NF_a (~4 mg) was reduced with sodium borohydride, permethylated, methanolyzed, and then submitted to g.l.c. analysis. Three peaks (*a*, *b*, and *c*) having a relative retention time of 1, 3.1, and 7.1 were observed. The identity of peaks *a* and *c* as methyl 2,3,4,6-tetra-*O*-methyl-D-mannopyranoside and 2,4-di-*O*-methyl-D-mannopyranoside, respectively, was established by comparison of the retention time with those of standards. Peak *b* corresponded to 2,5-anhydro-1,3,6-tri-*O*-methyl-D-mannitol. A 0.2mM solution of labelled and reduced tetrasaccharide was subjected to enzymic digestion with a commercial α-D-mannosidase (1 U/ml) obtained from *Turbo cornatus* (Seikagaku Kogyo Co Ltd., Chuo-Ku, Tokyo, Japan) in 0.05M citrate buffer, pH 4.2, for 18 h at 37°. The enzyme was destroyed by heating the digest at 100° for 1 min, and the hydrolyzate was analyzed by paper chromatography in solvent A. The sheet of Whatman No. 3 paper was cut into 1.0-cm strips and analyzed by scintillation spectrometry. Three peaks (*a*, *b*, and *c*) of radioactivity were detected in the ratio 1.0:4.0:2.1. Peak *a* corresponded to the original reduced oligosaccharide NF_a (**1**), whereas the faster-moving compounds *b* and *c* were enzyme-degraded oligosaccharides. G.l.c. of methanolyzed and trimethylsilylated compounds *b* and *c* suggested that the molar ratio of D-mannose to 2,5-anhydro-D-mannitol was 2.1:1.0 and 1.2:1.0, respectively. No free, labelled 2,5-anhydro-D-mannitol was detected. These results suggest the presence of 2 external α-D-mannose residues and corroborate previous reports[29,30]. The internal D-mannose residue linked to the 2,5-anhydro-D-mannitol was not hydrolyzed by the α-D-mannosidase. Moreover, enzymic degradation and methylation indicated that D-mannose residues were in pyranose form. Consequently, the partial structure **1** is proposed for compound NF_a.

1 α-D-Man*p*-(1→3)-[α-D-Man*p*-(1→6)]-β-D-Man*p*-(1→4)-2,5-anhydro-D-mannose
2 α-D-Man*p*-(1→3)-[α-D-Man*p*-(1→6)]-β-D-Man*p*-(1→4)-2-acetamido-2-deoxy-D-glucose
3 β-D-Gal*p*-(1→4)-2,5-anhydro-D-mannose
4 β-D-Gal*p*-(1→4)-2-acetamido-2-deoxy-D-glucose
5 Modified NeuNAc-(2→?)-[β-D-Gal*p*-(1→4)-2,5-anhydro-D-mannose]
6 NeuNAc-(2→?)-[β-D-Gal*p*-(1→4)-2-acetamido-2-deoxy-D-glucose]

Compound NF_b. On g.l.c., reduced fraction NF_b gave D-galactose and 2,5-anhydro-D-mannitol in the molar ratio 1.1:1.0. The reduced fraction (~3.5 mg) was permethylated and methanolyzed, and the products were submitted to g.l.c. analysis. Two major compounds were obtained. The first one was identified as a methyl 2,3,4,6-tetra-*O*-methyl-D-galactoside, thus confirming the presence of a D-galactopyranosyl residue at the nonreducing end of the oligosaccharide. The second compound having the highest retention time was identified as 2,5-anhydro-1,3,6-tri-*O*-methyl-D-mannitol, indicating that the 2,5-anhydro-D-mannose residue was linked at C-4.

Enzymic digestion of reduced fraction NF_b with a commercial β-D-galactosidase (1 U/ml) obtained from *Charonia lampas* (Seikagaku Kogyo Co Ltd., Chuo-Ku,

Tokyo, Japan) was performed in 0.01M phosphate buffer, pH 4.6, for 18 h at 37°. The release of D-galactose and 2,5-anhydro-D-mannitol was followed by g.l.c. of the trimethylsilyl derivatives. D-Galactose was almost completely removed by the enzyme and an equivalent amount of 2,5-anhydro-D-mannitol was obtained, indicating structure **3** for this disaccharide.

Fraction NF_c. On paper chromatography in solvent A, only one spot was detected either with the urea–phosphoric acid[27] or with the urea–hydrochloric acid reagent[26]. The rate of migration (R_{Gal} 0.98) was identical with that of 2,5-anhydro-D-mannose obtained by nitrous acid deamination of 2-amino-2-deoxy-D-glucose hydrochloride according to Bera *et al.*[17]. After reduction with an excess of sodium borohydride in aqueous solution for 20 h at 22°, the reduced compound NF_c was analyzed by g.l.c. of the trimethylsilyl ether. Only one peak was observed that showed a relative retention time (R_I 2.05, relative to erythritol) identical with that of synthetic 2,5-anhydro-D-mannitol[17].

Composition and structure of the oligosaccharides isolated from the acidic fraction AF_2. — The acidic fraction AF_2 was resolved by paper chromatography into four major components (Fig. 2C). Three acidic oligosaccharides, AF_{2a} (R_{Gal} 0.15), AF_{2b} (R_{Gal} 0.19), AF_{2c} (R_{Gal} 0.29), and one neutral oligosaccharide, AF_{2b} (R_{Gal} 0.61) were isolated in the respective yields of 4.5, 2.2, 1.0, and 1.1% by weight of the carbohydrate moiety of α_1-acid glycoprotein. Fractions AF_{2c} and AF_{2d} were isolated in quantities too small for the study of the structure. After elution from the chromatogram, fractions AF_{2a} and AF_{2b} were analyzed: each compound contained diphenylamine-positive material, D-galactose, and 2,5-anhydro-D-mannose. G.l.c. after reduction with sodium borohydride and methanolysis indicated 2,5-anhydro-D-mannitol and D-galactose in the molar ratio of 1:1. In addition, each oligosaccharide gave a peak eluted at 224° (R_I relative to erythritol: 4.3) on a OV-17 column; this compound was probably derived from the *N*-acetylneuraminic acid residues. Partial acid hydrolysis of compounds AF_{2a} and AF_{2b} (0.05M sulfuric acid, 1 h, 100°) gave a disaccharide (R_{Gal} 0.61) that had a ratio of D-galactose to 2,5-anhydro-D-mannose (1.1:1.0) identical with that of the NF_b compound **3**. Hence, the structure **5** is suggested for compounds AF_{2a} and AF_{2b}, which are derived from **6**.

DISCUSSION

As previously described[28], hydrazinolysis of α_1-acid glycoprotein cleaves 2-acetamido-1-*N*-(L-aspart-4-oyl)-2-deoxy-β-D-glucopyranosylamine linkages and gives de-*N*-acetylated polysaccharide chains (see Scheme 1). The complete de-*N*-acetylation of 2-acetamido-2-deoxy-D-glucose residues is necessary for a highly specific cleavage of 2-amino-2-deoxy-D-glucosyl bonds with sodium nitrite. The conditions of hydrazinolysis presently used (redistilled hydrazine, 30 h, 100°) for the scission of the amide groups of polysaccharide chains were those that had been shown to give the highest degree of de-*N*-acetylation for methyl 2-acetamido-2-deoxy-α-D-glucopyranoside[15,16]. The de-*N*-acetylated carbohydrate chains obtained by

hydrazinolysis of native α_1-acid glycoprotein were devoid of amino acids and were purified by fractionation on Sephadex G-50 (Fraction A, Fig. 1) with a yield of 80% in term of carbohydrate. Except a part of the neuraminosyl bonds, most of the glycosidic bonds of the carbohydrate chains were highly stable. Moreover, less than 2% of the 2-acetamido-2-deoxy-D-glucopyranose residues were found in Fraction A.

Nitrous acid deamination of the de-*N*-acetylated polysaccharide chains (Fraction A) gave, after fractionation on Dowex 1 (X-2), an acid (AF) and a neutral (NF) fraction. The latter one was fractionated by paper chromatography to give two neutral oligosaccharides (**1** and **3**) having 2,5-anhydro-D-mannose end-groups and free 2,5-anhydro-D-mannose, in the respective yields of 25, 25, and 6% in term of the native polysaccharide chains.

The neutral disaccharide NF_b has structure **3**, derives from **4**, and was obtained in a ratio of four moles per sialic acid-free carbohydrate unit and of only two moles per native carbohydrate unit (Fig. 3), because 50% of the *N*-acetylneuraminosyl bonds were stable and led to the formation of **5**. This result is in agreement with the partial structure obtained by enzymic degradation of sialic-free α_1-acid glycoprotein, four residues of D-galactose being first released by action of β-D-galactosidase[29,30], and then four residues of 2-acetamido-2-deoxy-D-glucose by action of *N*-acetyl-β-D-glucosaminidase[30,31]. Structure **4** is located at the peripheral part of the polysaccharide chains and has been previously described[2,6].

The neutral tetrasaccharide NF_a has structure **1**, derives from **2**, and was obtained with a ratio of one mole per sialic acid-free carbohydrate unit or native carbohydrate unit. It was shown[29,30] that the inner "core" of the carbohydrate chain, which consists of three residues of D-mannose and two residues of 2-acetamido-2-deoxy-D-glucose, is degraded with α-D-mannosidase to give two residues of D-mannose. Thus, it may be concluded that tetrasaccharide NF_a is derived from the inner part of the polysaccharide chain. Structure **1** agrees with the results of the permethylation[32,33] and with the two oligosaccharide structures, D-Man*p*-(1→3)-D-Man and D-Man*p*-(1→6)-D-Man obtained by partial acid hydrolysis of the whole glycoprotein[19]. Moreover, the occurrence of structure **2** has also been reported in a variety of proteins including IgE, IgM, IgG immunoglobulins[34–36], fetuin[15,37], ovomucoid[16], thyroglobulin[38], transferrin[15,39,40], and lactotransferrin[15,39], and in some oligosaccharides[41] accumulated in the liver of GM_1-gangliosidosis, type I.

Free 2,5-anhydro-D-mannose (NF_c) was isolated, in pure form, in the porportion of 0.8–1 mole per carbohydrate unit. Since no 2-amino-2-deoxy-3,4,6-tri-*O*-methyl-D-glucose was observed on permethylation of the native glycoprotein[4,32], this free 2,5-anhydro-D-mannose may derive from two associated internal residues of 2-acetamido-2-deoxy-D-glucose. On the other hand, it has been demonstrated[15] that hydrazinolysis followed by nitrous deamination of 2-acetamido-1-*N*-(L-aspart-4-oyl)-2-deoxy-β-D-glucosylamine gives almost quantitatively free 2,5-anhydro-D-mannose. Consequently, it is suggested that compound NF_c is derived from the 2-acetamido-2-deoxy-D-glucose residue linked to the asparagine residue.

The acidic fraction, which contains modified neuraminosyl residues, is derived

from the external part of the polysaccharide chains. The two acidic oligosaccharides AF_a and AF_b possess the same chemical composition and a partial common sequence. The complete structure of such compounds has been previously described[6,42].

Hydrazinolysis and nitrous acid deamination of a glycoprotein is a convenient procedure to obtain in a good yield a few well defined oligosaccharides, because 2-acetamido-2-deoxy-D-glucopyranosyl bonds are split off specifically. This is in contrast with other chemical methods of scission, such as partial hydrolysis or acetolysis, that produce numerous oligosaccharides.

ACKNOWLEDGMENTS

We are grateful to Professor R. W. Jeanloz for helpful advice in preparing this manuscript. We wish to thank Professeur J. Montreuil for valuable discussions. Expert technical assistance was provided by D. Roux and Y. Leroy. This work was supported by the Centre National de la Recherche Scientifique (L.A. n° 217), by the Commissariat à l'Énergie Atomique, and by the Institut National de la Santé et de la Recherche Médicale (U 124: Unité de recherches ultrastructurales et biochimiques sur les cellules normales et cancéreuses).

REFERENCES

1 I. YAMASHINA, *Acta Chem. Scand.*, 10 (1956) 1666–1672.
2 E. H. EYLAR AND R. W. JEANLOZ, *J. Biol. Chem.*, 237 (1962) 622–628.
3 B. FOURNET, G. TAKERKART, J. BROHON, AND J. MONTREUIL, *Bull. Soc. Chim. Biol.*, 50 (1968) 1352–1354.
4 B. FOURNET, Thèse Doctorat, Université des Sciences et Techniques, 1972, Lille, France.
5 B. Bayard and J. Montreuil, *Arch. Int. Physiol. Biochim.*, 77 (1969) 564–565.
6 M. ISEMURA AND K. SCHMID, *Biochem. J.*, 124 (1971) 591–604.
7 A. B. FOSTER, E. F. MARTLEW, AND M. STACEY, *Chem. Ind.* (London), (1953) 823–828.
8 Y. MATSUSHIMA AND N. FUJII, *Bull. Chem. Soc. Jap.*, 30 (1957) 48–50.
9 Z. YOSIZAWA AND T. SATO, *J. Biochem.* (Tokyo), 51 (1961) 233–241.
10 Z. YOSIZAWA AND T. SATO, *Biochim. Biophys. Acta*, 52 (1961) 591–593.
11 S. HASE AND Y. MATSUSHIMA, *J. Biochem.* (Tokyo), 72 (1972) 1117–1128.
12 J. K. N. JONES, P. REID, AND J. R. TURVEY, *Can. J. Chem.*, 43 (1965) 983–985.
13 D. HORTON, K. D. PHILIPS, AND J. DEFAYE, *Carbohydr. Res.*, 21 (1972) 417–419.
14 B. A. DMITRIEV, Y. KNIREL, AND N. K. KOCHETKOV, *Carbohydr. Res.*, 29 (1973) 451–457.
15 B. Bayard, Thèse Doctorat, Université des Sciences et Techniques, 1974, Lille, France.
16 B. BAYARD AND J. MONTREUIL, *Colloq. Int. Cent. Natl. Rech. Sci.*, 220 (1974) 209–218.
17 B. C. BERA, A. B. FOSTER, AND M. STACEY, *J. Chem. Soc.*, (1956) 4531–4535.
18 J. R. CLAMP, T. BHATTI, AND R. E. CHAMBERS, in A. GOTTSCHALK (Ed.), *Glycoproteins*, 2nd ed., Elsevier, Amsterdam, pp. 300–319.
19 B. FOURNET AND J. MONTREUIL, *J. Chromatogr.*, 75 (1973) 29–37.
20 W. BÜRGI AND K. SCHMID, *J. Biol. Chem.*, 236 (1961) 1066–1074.
21 R. DEDONDER, *Bull. Soc. Chim. Fr.*, 19 (1952) 864–879.
22 C. RIMINGTON, *Biochem. J.*, 34 (1940) 931.
23 R. BELCHER, A. J. NUTTEN, AND C. M. SAMBROOK, *Analyst* (London), (1954) 201–208.
24 I. WERNER AND L. ODIN, *Acta Soc. Med. Uppsal.*, 57 (1952) 230–241.
25 S. HAKOMORI, *J. Biochem.* (Tokyo), 55 (1964) 205–210.
26 D. HORTON AND K. D. PHILIPS, *Carbohydr. Res.*, 30 (1973) 367–374.
27 F. L. GREENE AND P. MORRIS, *Anal. Chem.*, 30 (1958) 1164–1170.
28 Z. YOSIZAWA, T. SATO, AND K. SCHMID, *Biochim. Biophys. Acta*, 121 (1966) 417–419.

29 K. M. L. AGRAWAL AND O. P. BAHL, *J. Biol. Chem.*, 243 (1967) 103–111.
30 V. B. HATCHER AND R. W. JEANLOZ, *Colloq. Int. Cent. Natl. Rech. Sci.*, 221 (1973) 329–338.
31 B. WEISSMANN, S. HADJIIOANNON AND J. TORNHEIN, *J. Biol. Chem.*, 239 (1964) 59–63.
32 G. SCHWARZMANN, V. REINHOLD, AND R. W. JEANLOZ, *Colloq. Int. Cent. Natl. Rech. Sci.*, 221 (1973) 85–94.
33 R. W. JEANLOZ, *Bull. Soc. Chim. Biol.*, 42 (1960) 303–325.
34 J. BAENZIGER, S. KORNFELD, AND S. KOCHWA, *J. Biol. Chem.*, 249 (1974) 1897–1903.
35 S. HICKMAN, R. KORNFELD, S. OSTERLAND, AND S. KORNFELD, *J. Biol. Chem.*, 247 (1972) 2156–2163.
36 R. KORNFELD, J. KELLER, J. BAENZIGER, AND S. KORNFELD, *J. Biol. Chem.*, 246 (1971) 3259–3268.
37 R. G. SPIRO, *J. Biol. Chem.*, 239 (1964) 567–573.
38 S. TOYOSHIMA, M. FUKUDA, AND T. OSAWA, *Biochem. Biophys. Res. Commun.*, 51 (1973) 945–950.
39 G. SPIK, R. VANDERSYPPE, B. FOURNET, B. BAYARD, P. CHARET, S. BOUQUELET, G. STRECKER, AND J. MONTREUIL, *Colloq. Int. Cent. Natl. Rech. Sci.*, 221 (1973) 383–399.
40 G. SPIK, B. BAYARD, B. FOURNET, G. STRECKER, S. BOUQUELET, AND J. MONTREUIL, *FEBS Lett.*, 50 (1975) 296–299.
41 L. S. WOLFE, R. G. SENIOR, AND N. M. K. YING KIN, *J. Biol. Chem.*, 249 (1974) 1828–1838.
42 R. W. JEANLOZ AND A. CLOSSE, *Fed. Proc.*, 22 (1963) 538.