Eur. J. Biochem. *180*, 101 – 110 (1989)

Analysis of *N*-acetyl-4-*O*-acetylneuraminic-acid-containing N-linked carbohydrate chains released by peptide-N^4-(*N*-acetyl-β-glucosaminyl)asparagine amidase F

Application to the structure determination of the carbohydrate chains of equine fibrinogen

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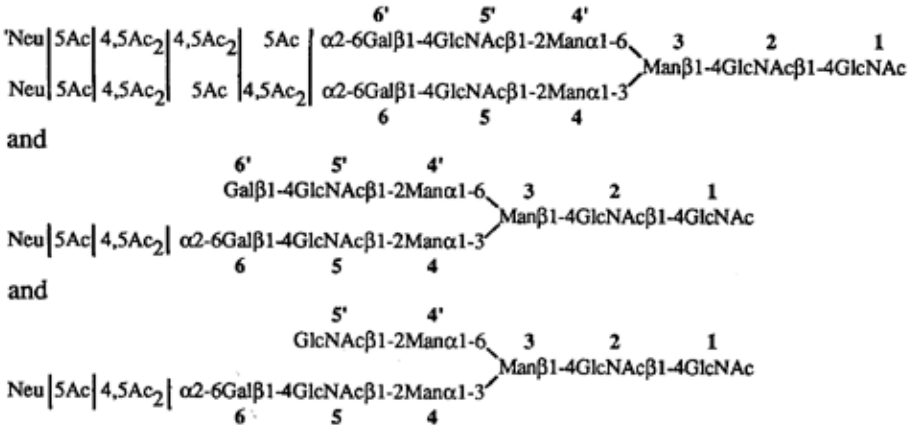
(Received July 29, 1988) – EJB 88 0920

The carbohydrate chains of equine fibrinogen were enzymatically released by peptide-N^4-(*N*-acetyl-β-glucosaminyl)asparagine amidase F. The oligosaccharides obtained were fractionated by a combination of FPLC and HPLC and analyzed by 500-MHz ^{1}H-NMR spectroscopy. Four monosialo and four disialo diantennary *N*-acetyllactosamine type of carbohydrate chains occur:



Sialic acids are widespread in animals as well as in micro-organisms. The amino function can be acetylated or glycolylated, whereas the hydroxyl functions may be acetylated, lactylated, methylated or sulfated [1]. Concerning the role of *O*-acetyl groups in sialic acids, information is accumulating about specific metabolic, biological and physico-chemical effects of these compounds [2]. In catabolic processes *O*-acetylation of sialic acids may hinder (7,8,9-OAc) or prevents (4-OAc) the action of sialidases, thereby influencing the lifetime of sialoglycoconjugates [3]. Furthermore, it has been found that *O*-acetylation influences the antigenicity of bacterial polysaccharides [4] and of human melanoma cells [5]. It has an effect on the capacity of human erythrocytes to activate the complement pathway [6], plays a role in environmental adaptations [7], and can be necessary for binding of viruses [8]. From the physico-chemical point of view, *O*-acetyl groups may cause conformational change of glycoproteins and they may reduce the hydrophilic properties of sialic acids [9, 10]. Because of these observations, interest in sialic acids bearing *O*-acetyl groups (and the carbohydrate chains carrying them) has increased in the past few years. In general, the presence and location of substituents in sialic acid residues in glycoproteins is determined after cleavage of these residues from the carbohydrate chain by mild acid hydrolysis or sialidase treatment [11]. The released sialic acids are analyzed by TLC, HPLC and/or GLC-MS. In the latter case the characterization is based on a series of highly specific fragment ions present in the electron-impact (EI) spectra of the trimethylsilylated sialic acid methyl or trimethylsilyl esters [12]. However, during mild acid treatment some of the *O*-acyl groups are split off, so that the analytical results do not reflect the native situation in the carbohydrate chain. Enzymatic release of 4-*O*-acetylated neuraminic acids is not feasable since the glycosidic bond is resistant to sialidases. A further complication in the analysis of sialic acids is the occurrence of migration of *O*-acetyl groups in the glycerol side chain, as has been demonstrated by ^{1}H-NMR spectroscopy [9, 13].

To obtain suitable products for the characterization of N-linked carbohydrate chains, several cleavage procedures are in use [14]. The chemical procedures are not especially

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Abbreviations. PNGase-F, peptide-N^4-(*N*-acetyl-β-glucosaminyl)asparagine amidase F; Neu5Ac, *N*-acetylneuraminic acid; Neu5Gc, *N*-glycolylneuraminic acid; Neu4,5Ac$_2$, *N*-acetyl-4-*O*-acetylneuraminic acid.

Enzymes. Peptide-N^4-(*N*-acetyl-β-glucosaminyl)asparagine amidase-F (EC 3.5.1.52); acyl-neuraminyl hydrolase, sialidase (EC 3.2.1.18).

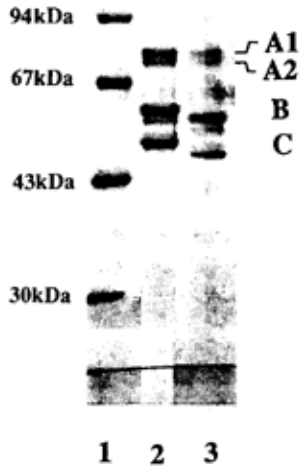


Fig. 1. *SDS/PAGE of equine fibrinogen on a 10% slab gel before and after treatment with PNGase-F.* Sample size 5–15 μg, staining with Coomassie brilliant blue. Lane 1, low-molecular-mass markers (phosphorylase *b*, 94 kDa; bovine serum albumin, 67 kDa; ovalbumin, 43 kDa; carbonic anhydrase, 30 kDa). Lane 2, equine fibrinogen, native (A1 + A2, α chains; B, β chain; C, γ chain). Lane 3, equine fibrinogen treated with PNGase-F

suitable when attention has to be paid to the *N*- and *O*-acyl substitution pattern of the sialic acid residues in the carbohydrate chain. In this respect the recent availability of an enzymatic method using peptide-N^4-(*N*-acetyl-β-glucosaminyl)asparagine amidase F (PNGase-F) to release N-linked carbohydrate chains [15–17] opens new perspectives. Here, the application of this enzyme in relation to conservation of sialic acid residues in N-linked chains will be shown for equine fibrinogen having Neu5Ac and Neu4,5Ac_2 residues as constituents of the carbohydrate chains.

MATERIALS AND METHODS

Materials

Equine fibrinogen (more than 80% clottable) was obtained from Sigma, St. Louis, USA. Peptide-N^4-(*N*-acetyl-β-glucosaminyl)asparagine amidase-F (PNGase-F) from *Flavobacterium meningosepticum* was obtained from Boehringer Mannheim, FRG, and was free of contaminating exoglycosidases.

Liberation of the carbohydrate chains

The purity of the fibrinogen sample was checked by SDS/PAGE [18] on a 10% slab gel, giving rise to four major bands (Fig. 1), marked A1, A2, B and C. The molecular masses were determined (Ferguson plot) to be 80.4 kDa, 76.5 kDa, 59.8 kDa and 51.2 kDa, respectively. The bands marked B and C represent the β and γ chains, respectively [19]. In analogy to human fibrinogen [19–22], the α chain is heterogeneous, giving rise to two α bands in SDS/PAGE differing by 3.9 kDa in molecular mass (A1, 80.4 kDa and A2, 76.5 kDa). The N-linked carbohydrate chains were released from the protein moiety essentially as described earlier [17], the major difference being that in this case the free enzyme was used. Briefly, 60 mg equine fibrinogen were dissolved in 4 ml 50 mM Tris, adjusted with concentrated HCl to pH 7.2, containing 10 mM EDTA, 1% (mass/vol.) SDS, and 1% (by vol.) 2-mercaptoethanol, and kept for 1 h at 40°C. The sample was diluted with 8 ml 50 mM Tris/HCl buffer, pH 7.2, containing 10 mM EDTA, and incubated with 16 U PNGase-F for 48 h at room temperature in an end-over-end mixer. SDS/PAGE and Coomassie brilliant blue staining shows (Fig. 1) that treatment of denatured equine fibrinogen with PNGase-F leads to a complete migration of the bands originally present at 59.8 kDa and 51.2 kDa to positions corresponding to apparent molecular masses of 57.5 kDa and 47.6 kDa, respectively. The bands belonging to the α chains show identical mobility before and after PNGase-F treatment. These data suggest that, similar to the human [23] and bovine [24] equivalents, only the β and γ chains are glycosylated, whereas the α chains are not. The differences in apparent molecular mass before and after PNGase-F treatment for the β (2.3 kDa) and γ (3.6 kDa) subunits suggest that each subunit contains only one glycosylation site, which is in agreement with the earlier reported carbohydrate content of 3% (by mass). The SDS/PAGE pattern after PNGase-F treatment indicates that removal of the carbohydrate chains from the β and γ subunits has proceeded to completion.

After desalting on a Bio-Gel P-2 column (1 × 18 cm, 200–400 mesh, Bio-Rad) and lyophilization, the sample was fractionated on a Bio-Gel P-6 column (1.9 × 50 cm, 200–400 mesh, Bio-Rad) using 0.05 M NH_4HCO_3, adjusted to pH 7 with HCl, as eluent. Carbohydrate-positive material (orcinol/H_2SO_4) was pooled and lyophilized. A second batch of 60 mg equine fibrinogen was worked up in the same way.

FPLC

Fractionation of the enzymatically released carbohydrate chains according to charge was carried out on a Mono Q HR 5/5 anion-exchange column (Pharmacia FPLC system), as described [17]. The fractionation was monitored at 214 nm and for quantification of the carbohydrates the number of C=O groups were taken into account. The carbohydrate fractions were collected, desalted and lyophilized.

HPLC

Subfractionation of the carbohydrate-containing FPLC fractions was carried out with a Kratos Spectroflow 400 HPLC system (Kratos Analytical, Rotterdam, The Netherlands) using a Lichrosorb-NH_2 10-μm column (25 × 0.46 cm, Chrompack, Middelburg, The Netherlands). Samples were dissolved in 0.1–0.2 ml 30 mM K_2HPO_4/KH_2PO_4, pH 7.0/acetonitrile (35:65, by vol.). Elutions were carried out isocratically with the same buffer at a flow rate of 120 ml/h at 25.0°C (SpH 99 column thermostat, Spark Holland B.V., Emmen, The Netherlands). Runs were monitored at 205 nm with a Spectroflow 783 programmable absorbance detector (Kratos Analytical) and peaks were integrated by a Spectra Physics SP 4290 integrator (Spectra Physics Inc., San José, California, USA). The corresponding HPLC fractions from the two working-up procedures were pooled and desalted on Bio-Gel P-2.

500-MHz ^{1}H-NMR spectroscopy

Prior to ^{1}H-NMR spectroscopic analysis the desalted samples were repeatedly treated with ^2H_2O, finally using 99.96 atom % ^2H_2O (Aldrich) at p^{2}H 7 and room temperature. Resolution-enhanced 500-MHz ^{1}H-NMR spectra were recorded using Bruker AM-500 spectrometers (Department of

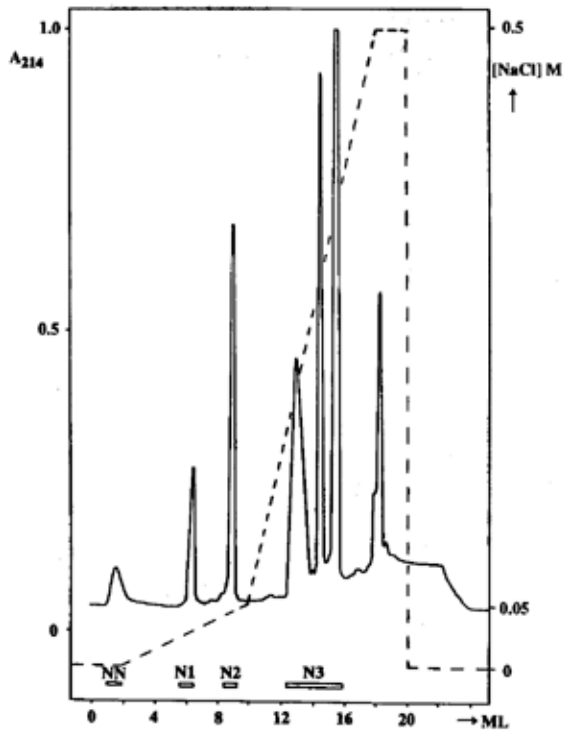


Fig. 2. *Fractionation pattern of the PNGase-F digestion products derived from equine fibrinogen on a FPLC HR 5/5 Mono Q column.* PNGase-F-treated equine fibrinogen was desalted, lyophilized and dissolved in 0.8 ml H_2O (HPLC quality). The column was eluted with a linear concentration gradient (----) of 0–50 mM NaCl in 8 ml H_2O (HPLC quality), followed by a steeper gradient of 50–500 mM NaCl in 8 ml H_2O at a flow rate of 60 ml/h. Injection volume 0.1 ml. Fractions were collected as indicated

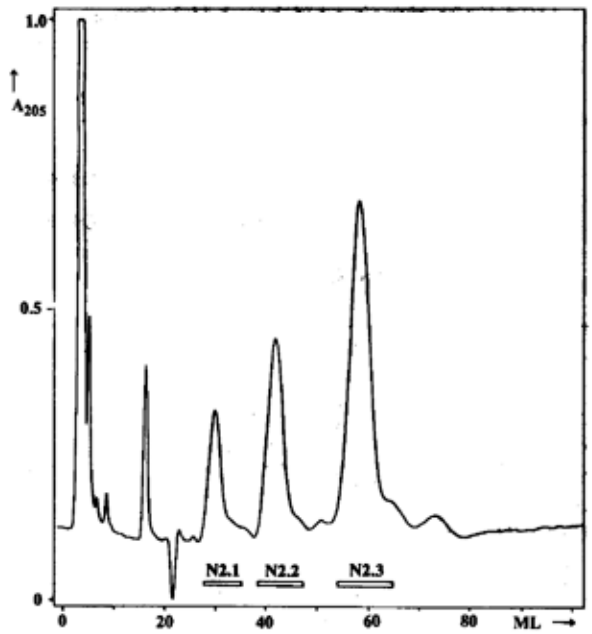


Fig. 3. *Fractionation pattern of the equine fibrinogen FPLC fraction N2 on a HPLC Lichrosorb-NH_2 10-μm column (25 × 0.46 cm, Chrompack).* The FPLC fraction was lyophilized, desalted and dissolved in 0.2 ml 30 mM potassium phosphate, pH 7.0/acetonitrile (35:65, by vol.). The column was eluted isocratically with the same buffer at a flow rate of 120 ml/h at 25.0°C. Injection volume 0.1 ml. Fractions were collected as indicated

NMR Spectroscopy, Utrecht University, and SON hf-NMR facility, Department of Biophysical Chemistry, Nijmegen University, The Netherlands) at a probe temperature of 27°C. Chemical shifts are expressed in ppm downfield from internal sodium 4,4-dimethyl-4-silapentane-1-sulphonate, but were actually measured by reference to internal acetone (δ = 2.225 ppm in ^2H_2O at 27°C) with an accuracy of 0.002 ppm [25].

Sugar analysis

Sugar analysis was carried out by gas-liquid chromatography on a capillary CP-Sil 5 WCOT fused silica column (25 m × 0.34 mm internal diameter, Chrompack, Middelburg, The Netherlands) using a Varian Aerograph 3700 gas chromatograph. The trimethylsilylated methyl glycosides were prepared by methanolysis, *N*-(re)acetylation and trimethylsilylation [12].

TLC-identification of Neu5Ac and Neu4,5Ac_2

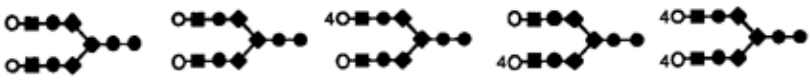
Equine fibrinogen (100 mg) was dissolved in 10 ml H_2O and dialyzed exhaustively against H_2O. Subsequently, the solution was lyophilized and the residue was resuspended in 10 ml 0.1 M HCl and incubated for 1 h at 80°C. Released Neu5Ac and Neu4,5Ac_2 were collected by dialysis against H_2O and lyophilization of the diffusate. The sialic acids were extracted with methanol and after evaporation purified by anion-exchange chromatography on Mono Q HR 5/5 (Pharmacia FPLC system) applying a linear concentration gradient of 0–50 mM NaCl in 8 ml H_2O, followed by a gradient of 50–500 mM NaCl in 8 ml H_2O [17]. The sialic-acid-containing fraction was collected and, after lyophilization, the sialic acids were dissolved in methanol and analyzed by TLC on plastic-coated silica 60 plates using propan-1-ol/H_2O (7:3, by vol.) as eluent [26]. Sialic-acid-containing spots were visualized by spraying with orcinol/Fe^{3+} and subsequent heating of the plates for 15 min at 120°C [26].

RESULTS

Analysis of the sugar constituents of equine fibrinogen demonstrates that it contains Gal, Man, GlcNAc and Neu5Ac in the molar ratio of 2.1:3.0:3.9:1.2; this is indicative for the occurrence of mono- and disialo diantennary *N*-acetyllactosamine type of carbohydrate chains. Treatment of the glycoprotein with 0.1 M HCl for 1 h at 80°C and subsequent TLC on silica plates of the isolated sialic acid constituents revealed the presence of a considerable amount of *O*-acetylated sialic acid (R_F = 0.47) in addition to Neu5Ac (R_F = 0.39) (results not shown). Medium-pressure anion-exchange chromatography of PNGase-F-digested equine fibrinogen over Mono Q gave rise to two oligosaccharide-containing fractions, denoted N1 and N2, and additional peaks, denoted NN and N3 (Fig. 2). Sugar analysis shows that N3 and NN do not contain carbohydrate material. N3 represents *N*-deglycosylated protein and remaining traces of SDS and Tris, whereas the identity of NN is unclear. Fraction N1 has the same elution volume as a reference monosialo monoantennary oligosaccharide obtained from human chorionic gonadotropin [17], whereas the elution volume of fraction N2 is identical to that of disialo diantennary reference compounds isolated from human serotransferrin and human chorionic gonadotropin [17]. HPLC of fraction N2 gives rise

Table 1. *^{1}H-chemical shifts of structural-reporter-group protons of the constituent monosaccharides for the oligosaccharides N2.1, N2.2A, N2.2B and N2.3 derived from equine fibrinogen, together with those for reference compound HST [17] released from human serotransferrin*

Chemical shifts are given at 300 K in ppm downfield from internal sodium 4,4-dimethyl-4-silapentane-1-sulphonate in ^2H_2O, but were actually measured by reference to internal acetone (δ = 2.225 ppm in ^2H_2O at 300 K). Compounds are represented by short-hand symbolic notation [25]: ●, GlcNAc; ◆, Man; ■, Gal; ○, Neu5Acα2-6; 4○, Neu4,5Ac$_2$α2-6

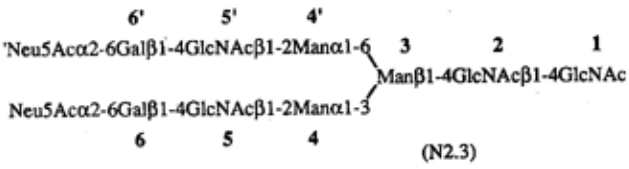


Reporter group	Residue[1]	HST	N2.3	N2.2B	N2.2A	N2.1
H-1	GlcNAc-1	α5.190, β4.696	α5.189, β4.694	α5.190, β4.694	α5.190, β4.694	α5.189, β4.696
	GlcNAc-2	α4.615, β4.606	α4.613, β4.604	α4.614, β4.606	α4.614, β4.606	α4.615, β4.609
	Man-3	n.d.	n.d.	~4.77[2]	~4.77[2]	n.d.
	Man-4	5.134	5.133	5.135	5.143	5.144
	Man-4'	4.947	4.948	4.956	4.950	4.957
	GlcNAc-5	4.606	4.604	4.606	4.606	4.609
	GlcNAc-5'	4.606	4.604	4.606	4.606	4.609
	Gal-6	4.443	4.443	4.444	4.440	4.440
	Gal-6'	4.447	4.447	4.444	4.447	4.442
H-2	Man-3	4.256	4.258	4.260	4.260	4.261
	Man-4	4.198	4.197	4.197	4.204	4.204
	Man-4'	4.118	4.117	4.124	4.117	4.125
H-3a	Neu5Ac	1.718	1.719	1.719	--	--
	Neu5Ac'	1.718	1.719	--	1.719	--
	Neu4,5Ac$_2$	--	--	--	1.852	1.852
	Neu4,5Ac$_2$'	--	--	1.852	--	1.852
H-3e	Neu5Ac	2.668	2.667	2.667	--	--
	Neu5Ac'	2.676	2.673	--	2.673	--
	Neu4,5Ac$_2$	--	--	--	2.677	2.677
	Neu4,5Ac$_2$'	--	--	2.684	--	2.682
H-4	Neu5Ac	n.d.	n.d.	3.660[3]	--	--
	Neu5Ac'	n.d.	n.d.	--	3.660[3]	--
	Neu4,5Ac$_2$	--	--	--	4.904	4.903
	Neu4,5Ac$_2$'	--	--	4.904	--	4.903
H-5	Neu4,5Ac$_2$	--	--	--	4.052[4]	4.052
	Neu4,5Ac$_2$'	--	--	4.052[4]	--	4.052
NAc	GlcNAc-1	2.037	2.038	2.038	2.038	2.037
	GlcNAc-2	2.083	2.084	2.085	2.085	2.085
	GlcNAc-5	2.069	2.070	2.070	2.110	2.110
	GlcNAc-5'	2.066	2.066	2.104	2.066	2.104
	Neu5Ac	2.030	2.029	2.030	--	--
	Neu5Ac'	2.030	2.029	--	2.030	--
	Neu4,5Ac$_2$	--	--	--	1.964	1.964
	Neu4,5Ac$_2$'	--	--	1.964	--	1.964
OAc	Neu4,5Ac$_2$	--	--	--	2.077	2.077
	Neu4,5Ac$_2$'	--	--	2.077	--	2.077

[1] For numbering of the monosaccharide residues, see text.
[2] Value obtained at 310 K.
[3] Value obtained by selective irradiation of Neu5Ac H-3a at 310 K.
[4] Value obtained by selective irradiation of Neu4,5Ac$_2$ H-4 at 310 K. n.d.: not determined.

to three subfractions, denoted N2.1 ($R_{N2.1} = 0.52 \times R_{N2.3}$), N2.2 ($R_{N2.2} = 0.72 \times R_{N2.3}$) and N2.3 ($R_{N2.3} = 1.00$), respectively (Fig. 3). Fraction N2.3 has the same elution position as the α2-6-disialylated diantennary reference oligosaccharide HST (see Table 1). HPLC of fraction N1 yields also three subfractions, denoted N1.1 ($R_{N1.1} = 0.56 \times R_{N1.3}$), N1.2 ($R_{N1.2} = 0.76 \times R_{N1.3}$) and N1.3 ($R_{N1.3} = 1.00$), respectively (Fig. 4).

For structural analysis fractions N2.1-N2.3 and N1.1-N1.3 were investigated by 500-MHz ^{1}H-NMR spectroscopy and they will be discussed in order of complexity. The ^{1}H-NMR data of fractions N2.1-N2.3, together with those of reference compound HST, are compiled in Table 1. The ^{1}H-NMR spectrum of fraction N2.3 (Figs 5A and 6A) indicates the following diantennary structure containing two α2-6-linked Neu5Ac residues:



The various structural-reporter-group signals of N2.3 match completely those of reference oligosaccharide HST (Table 1). It has to be noted that the presence of the usual *N,N'*-diacetylchitobiosyl-trimannosyl core as part of a diantennary type of carbohydrate chain is reflected by the characteristic set of H-1 and NAc signals of GlcNAc-**1** and GlcNAc-**2** and the H-1 and H-2 structural-reporter-group signals of Man-**3**, Man-**4** and Man-**4'** [17, 25].

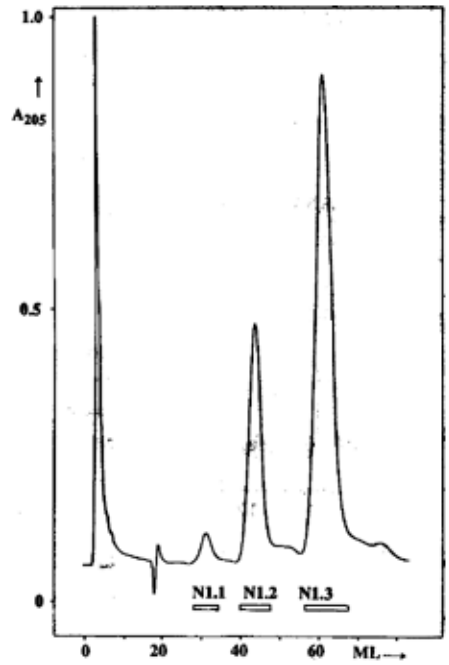
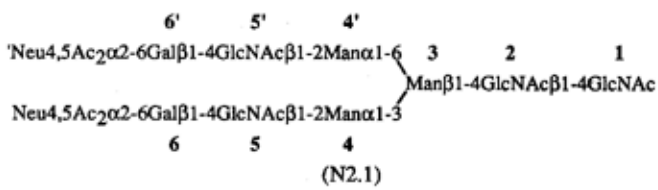


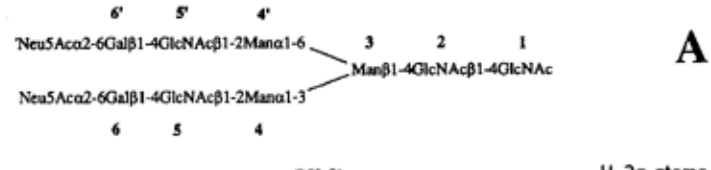
Fig. 4. *Fractionation pattern of the equine fibrinogen FPLC fraction N1 on a HPLC Lichrosorb-NH₂ 10-μm column (25 × 0.46 cm, Chrompack).* The FPLC fraction was lyophilized, desalted and dissolved in 0.1 ml 30 mM potassium phosphate, pH 7.0/acetonitrile (35/65, by vol.). For further details see Fig. 3

The ^{1}H-NMR spectrum of fraction N2.1 (Figs 5C and 6C) shows that it contains essentially the same structure as N2.3, except that both α2-6-linked Neu5Ac residues are *O*-acetylated at C-4:



(N2.1)

Similar to N2.3, in N2.1 the H-1 signals of Man-**4** and Man-**4'** at δ = 5.144 ppm and δ = 4.957 ppm, respectively, reflect the occurrence of α2-6-linked sialic acid residues in both branches. The α2-6-linked Neu4,5Ac$_2$ residues show a different set of structural-reporter-group signals, namely, H-3a at δ = 1.852 ppm, H-3e at δ = 2.677/2.682 ppm, H-4 at δ = 4.903 ppm, H-5 at δ = 4.052 ppm, NAc (5Ac) at δ = 1.964 ppm and OAc (4Ac) at δ = 2.077 ppm (see irradiation studies for the confirmation of the assignments, N2.2). Guided by the Neu5Ac H-3e reporters in N2.3, the H-3e signals at δ = 2.677 and δ = 2.682 ppm were assigned to Neu4,5Ac$_2$ in the Manα1-3 and Manα1-6 branch, respectively. The values of the coupling constants $J_{3e,4}$ (4.6 Hz), $J_{3a,3e}$ (−12.2 Hz) and $J_{3a,4}$ (12.2 Hz) in Neu4,5Ac$_2$ are comparable to those found in Neu5Ac [10]. Comparison of the Neu5Ac and Neu4,5Ac$_2$ structural-reporter sets for α2-6-linked residues shows that the introduction of the electron-withdrawing *O*-acetyl group at C-4 of Neu5Ac results in downfield shifts for the Neu4,5Ac$_2$ H-3a ($\Delta\delta$ = +0.133 ppm), H-3e ($\Delta\delta$ = +0.010/+0.009 ppm), and H-4 ($\Delta\delta$ = +1.243 ppm; see N2.2) signals and in an upfield shift for the NAc signal ($\Delta\delta$ = −0.065 ppm).



A

(N2.3)

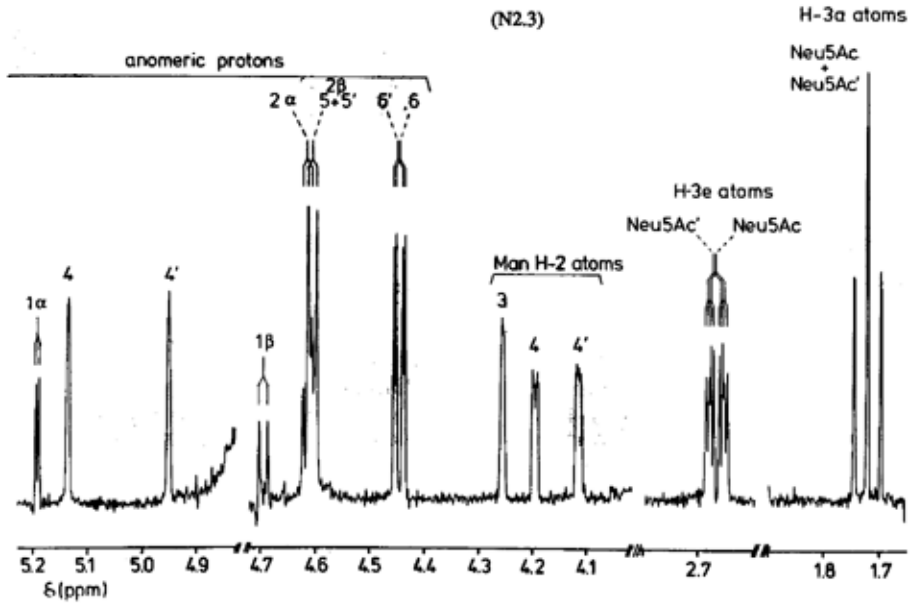


Fig. 5. *Resolution-enhanced 500-MHz ^{1}H-NMR spectra of the structural-reporter-group regions of oligosaccharides N2.1–3 obtained from equine fibrinogen.* The spectra were measured at 300 K in ^2H_2O. The acetyl region is not shown. (A) N2.3; (B) N2.2A and N2.2B; (C) N2.1

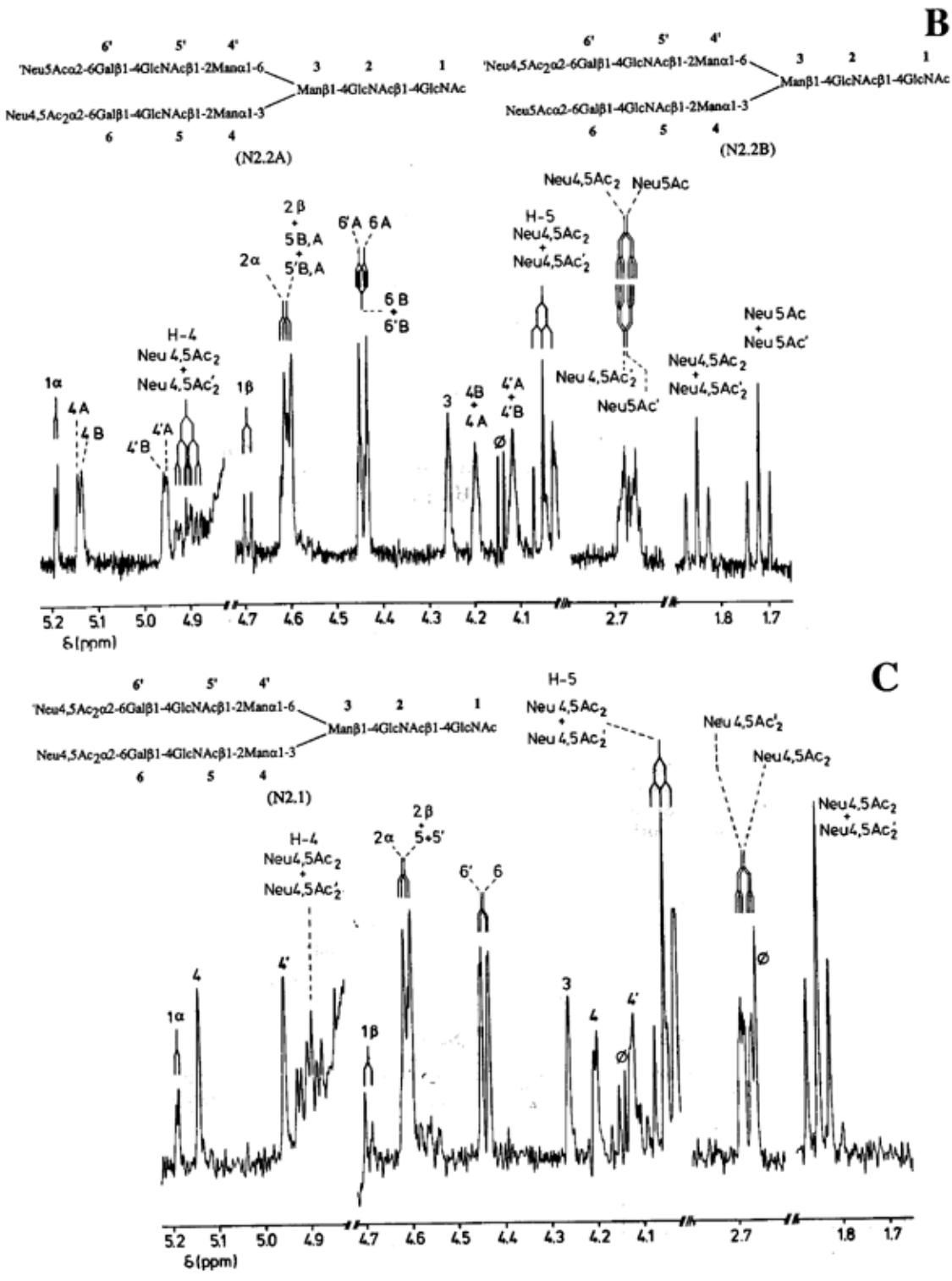


Fig. 5

The various shift increments are comparable to the 4-*O*-acetylation effects known for α2-3-linked sialic acid. Going from Neu5Acα2-3Galβ1-4Glc to Neu4,5Ac$_2$α2-3Galβ1-4Glc [9], downfield shifts were observed for H-3a ($\Delta\delta$ = +0.127 ppm), H-3e ($\Delta\delta$ = +0.011 ppm) and H-4 ($\Delta\delta$ = +1.267 ppm) and an upfield shift for the NAc singlet ($\Delta\delta$ = −0.067 ppm). The OAc signal in the latter compound is present at δ = 2.070 ppm. The presence of the Neu4,5Ac$_2$ residue also has a definite influence on the structural-reporter groups of the two branches. Comparison of the ^{1}H-NMR data of the Manα1-6 branches of N2.3 and N2.1 demonstrates the following effects: Man-**4′**: H-1, $\Delta\delta$ = +0.009 ppm; H-2, $\Delta\delta$ = +0.008 ppm; GlcNAc-**5′**: H-1, $\Delta\delta$ = +0.005 ppm, $J_{1,2}$ = 7.8 Hz; NAc, $\Delta\delta$ = +0.038 ppm (see Fig. 6C); and Gal-**6′**: H-1, $\Delta\delta$ = −0.005 ppm, $J_{1,2}$ = 7.8 Hz. For the Man α1-3 branch similar effects were found: Man-**4**: H-1, $\Delta\delta$ = +0.011 ppm; H-2, $\Delta\delta$ = +0.007 ppm; GlcNAc-**5**: H-1, $\Delta\delta$ = +0.005 ppm, $J_{1,2}$ = 7.8 Hz; NAc, $\Delta\delta$ = +0.040 ppm (see Fig. 6C); and Gal-**6**: H-1, $\Delta\delta$ = −0.003 ppm, $J_{1,2}$ = 7.8 Hz. It has to be noted that the assignments of the NAc signals of GlcNAc-**5** (δ = 2.110 ppm) and GlcNAc-**5′** (δ = 2.104 ppm) are based on the analysis of fraction N1.2 (see below).

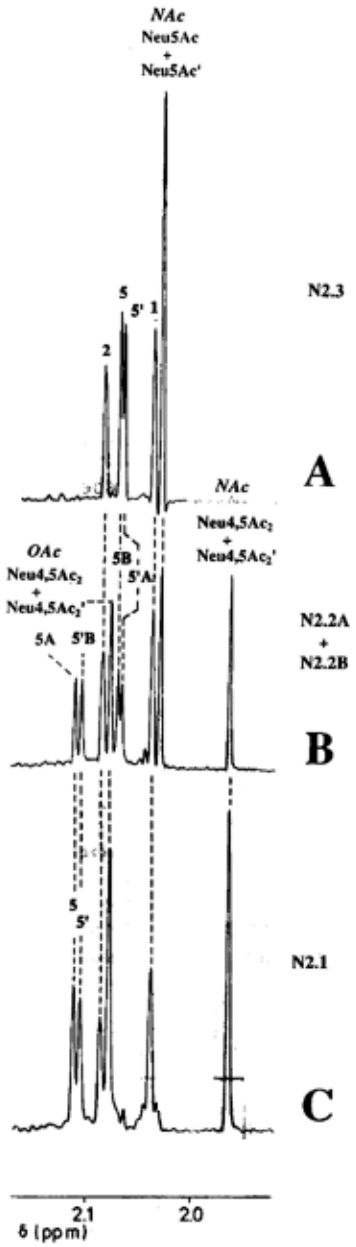
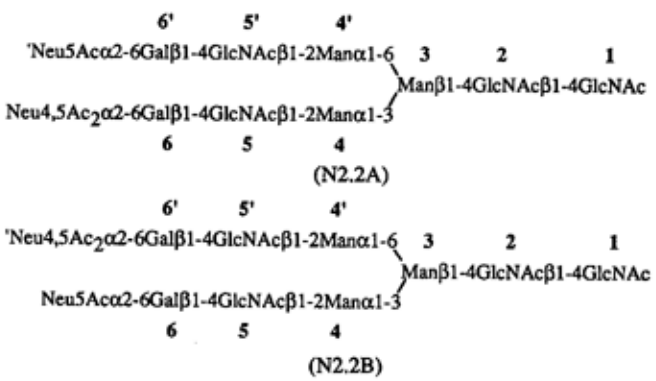


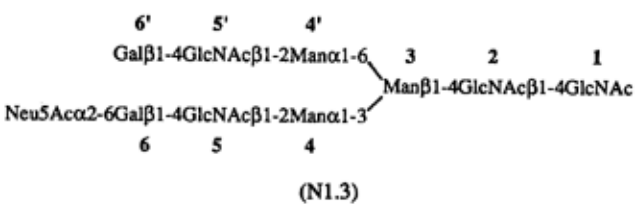
Fig. 6. *Acetyl regions from the resolution-enhanced 500-MHz ^{1}H-NMR spectra of the oligosaccharides N2.1–3 obtained from equine fibrinogen.* Details as in Fig. 5

The ^{1}H-NMR spectrum of fraction N2.2 (Figs 5B and 6B) demonstrates the presence of an equimolar mixture of the compounds N2.2A and N2.2B (Table 2):



Comparison of the ^{1}H-NMR spectrum of N2.2A/N2.2B with those of N2.1 and N2.3 reveals that N2.2A represents a diantennary oligosaccharide having Neu5Ac in terminal position of the Manα1-6 branch and Neu4,5Ac$_2$ in terminal position of the Manα1-3 branch, whereas N2.2B represents the equivalent carrying Neu5Ac and Neu4,5Ac$_2$ in terminal position of the Manα1-3 and Manα1-6 branches, respectively. The presence of Neu4,5Ac$_2$, α2-6-linked to Gal-**6**/**6**′, is evident from its set of structural reporters at δ = 1.852 ppm (H-3a), δ = 2.677/2.684 ppm (H-3e), δ = 4.904 ppm (H-4), δ = 1.964 ppm (NAc) and δ = 2.077 ppm (OAc). Selective irradiation of the H-3a signal of Neu4,5Ac$_2$ confirmed the assignments of the H-4 and H-3e signals of Neu4,5Ac$_2$, whereas selective irradiation of the H-4 signal (at 310 K) showed the H-5 signal to resonate at δ = 4.052 ppm. Likewise, the presence of Neu5Ac in α2-6 linkage to Gal-**6**/**6**′ is demonstrated by its structural reporters at δ = 1.719 ppm (H-3a), δ = 2.667/2.673 ppm (H-3e) and δ = 2.030 ppm (NAc). Based on the relative intensities of the two sets of signals, it can be concluded that Neu4,5Ac$_2$ and Neu5Ac are present in equimolar amounts. The Neu4,5Ac$_2$ and Neu5Ac residues are evenly distributed over the Manα1-6 branch, as reflected by the presence of two sets of signals of identical intensity stemming from the Man-**4**′ and Gal-**6**′ residues. In the case of Neu4,5Ac$_2$ the signals of Man-**4**′ H-1, Man-**4**′ H-2 and Gal-**6**′ H-1 are found at δ = 4.956 ppm, δ = 4.124 ppm and δ = 4.444 ppm, respectively, whereas in case of Neu5Ac these signals occur at δ = 4.950 ppm, δ = 4.117 ppm and δ = 4.447 ppm, respectively. Similarly it can be deduced that Neu4,5Ac$_2$ and Neu5Ac are also evenly distributed over the Manα1-3 branch. The presence of Neu4,5Ac$_2$ is evidenced by Man-**4** H-1 at δ = 5.143 ppm, Man-**4** H-2 at δ = 4.204 ppm and Gal-**6** H-1 at δ = 4.440 ppm, and the occurrence of Neu5Ac by Man-**4** H-1 at δ = 5.135 ppm, Man-**4** H-2 at δ = 4.197 ppm and Gal-**6** H-1 at δ = 4.444 ppm. Because HPLC of fraction N2.2 gives rise to a single peak having a retention volume intermediate to that of N2.1 and N2.3, it can be ruled out that N2.2 represents an equimolar mixture of N2.1 and N2.3.

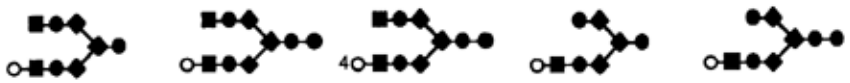
The ^{1}H-NMR data of fractions N1.1–N1.3, together with those of suitable reference compounds, are compiled in Table 2 and are discussed in order of complexity. The acetyl region of each fraction is presented in Fig. 7. The ^{1}H-NMR spectrum of fraction N1.3 demonstrates the presence of the following monosialo diantennary oligosaccharide:



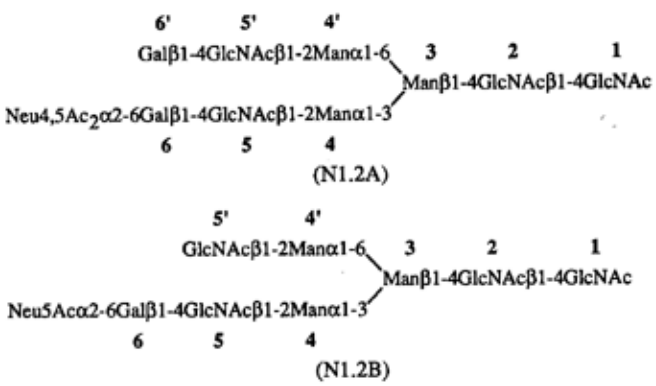
The presence of α2-6-linked Neu5Ac is indicated by its structural-reporter-group signals for H-3a (δ = 1.719 ppm), H-3e (δ = 2.668 ppm) and NAc (δ = 2.030 ppm) (compare with N2.3 and **27**). The latter sialic acid is present in the Manα1-3 branch at Gal-**6**, as proved by the H-1 signals of Man-**4**, GlcNAc-**5** and Gal-**6**. The H-1 signals of GlcNAc-**5**′ and Gal-**6**′ demonstrate the presence of terminal Gal-**6**′ in the Manα1-6 branch. The structural-reporters of both branches match completely those of reference compound **27** [25].

The ^{1}H-NMR spectrum of fraction N1.2 (Fig. 6B) reveals the presence of a mixture of two oligosaccharides, namely N1.2A and N1.2B:

Table 2. *^{1}H-chemical shifts of structural-reporter-group protons of the constituent monosaccharides for the oligosaccharides N1.2A, N1.2B and N1.3 derived from equine fibrinogen, together with those for reference compounds 25 and 27 [25]*
For further details see Table 1. For numbering of the monosaccharide residues, see text



Reporter group	Residue	27	N1.3	N1.2A	25	N1.2B
H-1	GlcNAc-1	--	α5.189, β4.695	α5.189, β n.d.	--	α5.189, β n.d.
	GlcNAc-2	α5.214, β~4.72	α4.614, β4.606	α4.614, β4.606	α5.213, β~4.72	α4.614, β4.606
	Man-3	α4.781, β4.771	n.d.	n.d.	α4.784, β4.776	n.d.
	Man-4	5.138	5.135	5.145	5.137	5.135
	Man-4'	α4.929, β4.932	4.930	4.930	4.921	4.919
	GlcNAc-5	4.608	4.606	4.606	4.607	4.606
	GlcNAc-5'	4.586	4.582	4.583	4.558	4.554
	Gal-6	4.446	4.446	4.444	4.446	4.446
	Gal-6'	α4.471, β4.473	4.473	4.474	--	--
H-2	Man-3	α4.260, β4.249	4.253	4.255	α4.264, β4.253	4.255
	Man-4	4.197	4.196	4.224	4.198	4.195
	Man-4'	4.114	4.112	4.110	4.109	4.106
H-3a	Neu5Ac	1.720	1.719	--	1.718	1.719
	Neu4,5Ac$_2$	--	--	1.851	--	--
H-3e	Neu5Ac	2.669	2.668	--	2.669	2.668
	Neu4,5Ac$_2$	--	--	2.678	--	--
NAc	GlcNAc-1	--	2.038	2.038	--	2.038
	GlcNAc-2	α2.061, β2.058	2.081	2.079	α2.059, β2.055	2.079
	GlcNAc-5	2.070	2.069	2.110	2.070	2.069
	GlcNAc-5'	α2.050, β2.048	2.047	2.048	2.053	2.052
	Neu5Ac	2.031	2.030	--	2.030	2.030
	Neu4,5Ac$_2$	--	--	1.964	--	--
OAc	Neu4,5Ac$_2$	--	--	2.077	--	--



The presence of both α2-6-linked Neu5Ac and Neu4,5Ac$_2$ is evident from the two sets of characteristic signals at δ = 2.030 ppm, δ = 2.668 ppm and δ = 1.719 ppm (NAc, H-3e and H-3a signals of Neu5Ac, respectively) and at δ = 1.964 ppm, δ = 2.077 ppm, δ = 2.678 ppm, and δ = 1.851 ppm (NAc, OAc, H-3e and H-3a signals of Neu4,5Ac$_2$, respectively). As calculated from the relative intensities of the specific NAc and H-3a signals, the molar ratio of Neu5Ac/Neu4,5Ac$_2$ amounts to 3:2. The chemical shift values of the H-1 signals of Man-**4** and Man-**4'** show that the α2-6-linked sialic acids exclusively occur in the Manα1-3 branch [25]. The presence of Neu5Ac in α2-6 linkage to Gal-**6** (N1.2B) is demonstrated by the H-1 signal of Man-**4** at δ = 5.135 ppm, whereas the occurrence of Neu4,5Ac$_2$ in α2-6 linkage to Gal-**6** (N1.2A) is indicated by the Man-**4** H-1 signal at δ = 5.145 ppm. The relative intensities of the Man-**4** H-1 signals also show a ratio of 3:2.

The Manα1-6 branch can be terminated with Gal-**6'** or GlcNAc-**5'**. The outer chain bearing Gal-**6'** in terminal position is reflected by the H-1 signals of GlcNAc-**5'** and Gal-**6'** (cf. N1.3 and **27**). The presence of an outer chain having GlcNAc-**5'** in terminal position is deduced from the typical GlcNAc-**5'** H-1 and NAc signals at δ = 4.554 ppm and δ = 2.052 ppm, respectively (compare reference compound **25**). Based on the relative peak areas of the GlcNAc-**5'** NAc signals at δ = 2.052 ppm (terminal) and δ = 2.048 ppm (internal) it can be concluded that the molar ratio of N1.2B relative to N1.2A is 3:2. The relative intensities of the two H-1 signals of Man-**4'** at δ = 4.930 ppm (N1.2A) and at δ = 4.919 ppm (N1.2B) are in agreement with this ratio. From the combination of these data it can be concluded that fraction N1.2 consists of a mixture of oligosaccharides N1.2B and N1.2A in a molar ratio of 3:2. Since in N1.2A the Neu4,5Ac$_2$ residue occurs exclusively on the Manα1-3 branch it is clear that the NAc signal at δ = 2.110 ppm stems from GlcNAc-**5**. Consequently, the NAc signal at δ = 2.110 ppm occurring in the

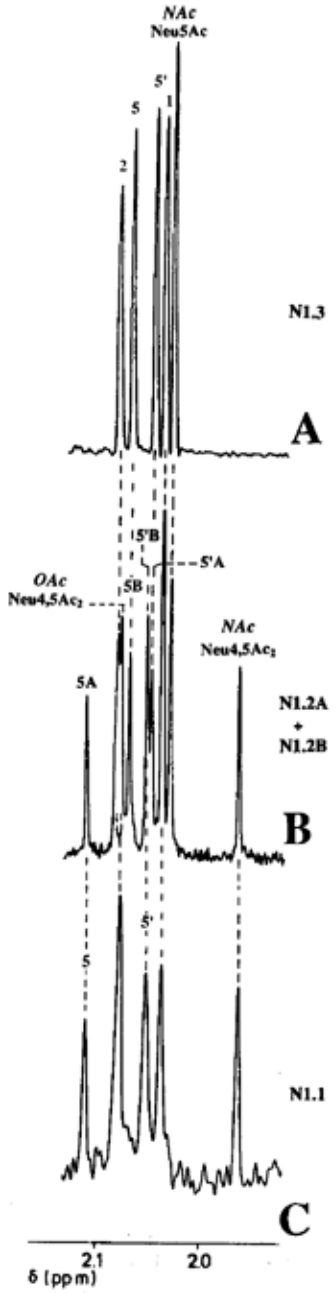
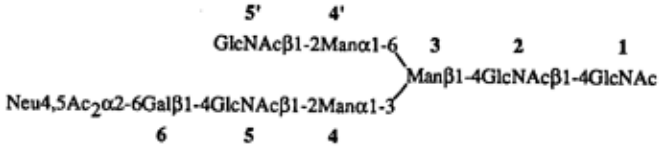


Fig. 7. *Acetyl regions from the resolution-enhanced 500-MHz ^{1}H-NMR spectra (^2H_2O; 300 K) of the oligosaccharides N1.1–3 (C, B and A) obtained from equine fibrinogen*

disialylated compounds N2.1 and N2.2A discussed previously (Table 1), belongs to GlcNAc-**5** and the NAc signal at δ = 2.104 ppm occurring in N2.1 and N2.2B, belongs to GlcNAc-**5′**.

Owing to the low amount of oligosaccharide material present in fraction N1.1, only the Ac region of the ^{1}H-NMR spectrum which shows five signals, with one of double intensity, could be analyzed (Fig. 6C). The presence of the *N,N′*-diacetylchitobiose unit is demonstrated by the NAc singlets at δ = 2.037 ppm and δ = 2.079 ppm, respectively. The Ac signals at δ = 2.109 ppm, δ = 1.964 ppm and δ = 2.077 ppm correspond with those of GlcNAc-**5** NAc, Neu4,5Ac$_2$ NAc and Neu4,5Ac$_2$ OAc, respectively, in N1.2A. The Ac signal at δ = 2.052 ppm is identical to that of GlcNAc-**5′** NAc in N1.2B. By combination of these data, it is obvious that fraction N1.1 has the following structure:



(N1.1)

HPLC of fractions N2 and N1 demonstrates that 4-*O*-acetylation of Neu5Ac results in a decrease of the relative HPLC retention volume with a factor 0.72 (N2.3 → N2.2A/N2.2B → N2.1, Fig. 3; and N1.3 → N1.2A, Fig. 4). The coelution of N1.2A and N1.2B in the HPLC separation of fraction N1 illustrates that substitution of a Neu5Ac residue by a Neu4,5Ac$_2$ residue and the absence of Gal-**6′** cancel each other. Since substitution of a Neu5Ac residue by a Neu4,5Ac$_2$ residue decreases the HPLC retention volume for diantennary oligosaccharides by a factor 0.72 it can be anticipated that $R_{N1.1} = R_{N1.2B} \times 0.72 = 0.55$. The observed value ($R_{N1.1}$ = 0.56, Fig. 4) is in close agreement, supplying additional evidence for the proposed structure.

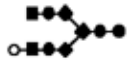
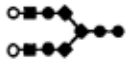
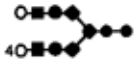
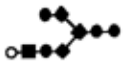
The relative molar amounts, based on their ultraviolet absorption, of the various carbohydrate chains occurring in equine fibrinogen are compiled in Table 3.

DISCUSSION

The occurrence of Neu4,5Ac$_2$ as a sialic acid constituent seems to be a general feature of equine glycoproteins [1, 4, 27]. In this paper it is demonstrated that the use of PNGase-F allows determination of Neu4,5Ac$_2$ on the free and intact oligosaccharide. However, it has to be noted that extrapolation of the results obtained to the situation *in vivo* is feasable only when, during the isolation of the glycoprotein, compatible conditions have also been employed. At present the suitability of PNGase-F in the study of N-linked oligosaccharides containing neuraminic acid residues bearing *O*-acetyl groups in the glycerol side chain or a *N*-glycolyl group, is under investigation.

In equine fibrinogen, as in bovine and human fibrinogen, mono- and disialo diantennary *N*-acetyllactosamine type of carbohydrate chains occur. In human fibrinogen the ratio of the disialo glycan relative to the monosialo glycan is approximately 2:1 [23], whereas in bovine fibrinogen this ratio appears to be 1:1 [24]. In human and bovine fibrinogen the carbohydrate chains occur exclusively on the β and γ subunits, each subunit bearing one oligosaccharide chain. It is assumed that the monosialo glycans are preferentially located on the γ subunit, whereas the disialo glycans are mainly located on the β subunit. In this report it is demonstrated that in equine fibrinogen the ratio of monosialo to disialo diantennary carbohydrate chains is approximately 2:3. The electrophoretic behaviour on polyacrylamide slab gels, before and after removal of the carbohydrate chains from the polypeptide moieties, indicates that the equine β and γ subunits each bear one oligosaccharide. The distribution over the subunits, with respect to the type of carbohydrate chain, remains to be clarified.

Table 3. *Carbohydrate chains occurring in equine fibrinogen*
The oligosaccharides were released by treatment with PNGase-F, fractionated by subsequent FPLC and HPLC and analyzed by 500-MHz ^{1}H-NMR spectroscopy. The molar ratios of oligosaccharides are given as percentages (total amount 100%). For quantification of the oligosaccharides the following procedure holds. (a) The molar ratio of oligosaccharides present in the FPLC fractions N1 and N2 is determined on basis of the number of C=O groups (absorbance at 214 nm) being known after structural identification. (b) The molar ratio of the constituent oligosaccharides within each FPLC fraction is determined on the basis of HPLC peak areas (corrected for the number of C=O groups) at 205 nm

Monosialooligosaccharide		Molar ratio	Disialooligosaccharide		Molar ratio
		%			%
	N1.3	27		N2.3	36
	N1.2A	4		N2.2A	9
	N1.2B	6		N2.2B	9
	N1.1	1		N2.1	8
Total		38			62

This work was supported by the Netherlands Foundation for Chemical Research (SON) with financial aid from the Netherlands Organization for Scientific Research (NWO) and the Netherlands Innovation Directed Research Program on Carbohydrates (IOP-K).

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