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Structure determination of the major N- and O-linked carbohydrate chains of the β subunit from equine chorionic gonadotropin

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The carbohydrate moieties of equine chorionic gonadotropin α and β subunits were released from the protein backbones by successive treatments with peptide-N^4-(*N*-acetyl-β-glucosaminyl)asparagine amidase F and alkaline borohydride and then fractionated by FPLC and HPLC. The major N- and O-linked glycans of the β subunit were characterized by 500-MHz ^{1}H-NMR spectroscopy, showing a remarkable structural heterogeneity for the *N*-glycosidically linked chains, comprising mono-, di-, tri- and tri′-antennary *N*-acetyllactosamine type of glycans, being partly α1-6 fucosylated at the Asn-bound GlcNAc residue and having α2-6 and α2-3 linked *N*-acetyl- and *N*-acetyl-4-*O*-acetylneuraminic acid residues as sialic acid constituents. Significant differences in this respect were detected for the partially characterized glycans of the α subunit. The major part of the O-linked carbohydrate chains, occurring solely in the β subunit, is formed by tri-, tetra-, penta- and hexa-saccharides. There are indications for the presence of oligo(*N*-acetyllactosamine) units in both the N- and O-linked glycans of the β subunit.

Equine chorionic gonadotropin (eCG) is a glycoprotein hormone synthesized and secreted by specialized cells derived from the fetal trophoblast [1]. The glycoprotein consists of two dissimilar, non-covalently linked subunits, designated α and β [2], having molecular masses of approximately 17 kDa and 44 kDa, respectively [3]. In the mare eCG shows LH activity and in this respect it is analogous to human CG [4]. In immature rats, however, eCG exhibits both follicle-stimulating-hormone and luteinizing-hormone (LH) activities [5]. This dual activity, the basis of which still remains to be clarified, was also demonstrated *in vitro* using highly purified preparations of eCG [3, 6].

Among all glycoprotein hormones, eCG has the highest content of carbohydrate, forming approximately 40% (by mass) and consisting of L-Fuc, D-Man, D-Gal, D-GlcNAc, D-GalNAc and sialic acid [3]. The major part of the carbohydrate is associated with the β subunit and, like hCG-β and eLH-β, eCG-β possesses a heavily *O*-glycosylated COOH-terminal extension of about 25 amino acids, which is linked by an acid-labile peptide bond at position 120–121 [7]. Recently, it was suggested that eCG-β carries six O-linked carbohydrate chains, part of them being very large with poly(*N*-acetyllactosamine) peripheral extensions, forming about 85% of the carbohydrate material of the β subunit [8] and one N-linked carbohydrate chain ranging from di- to penta-antennary in structure [9]. The N-linked carbohydrates of the α subunit seem to be smaller [10]. The distribution with regard to the type of carbohydrate chains (*N*- or *O*-glycosidically linked) over α and β subunits is similar to that in hCG; however, it is clear that there are also definite dissimilarities between hCG and eCG, both in protein [11, 12] and in carbohydrate moieties. Moreover, eCG contains one sulfate group [13]. Additional indications for structural and functional differences are the inability of eCG-β to combine with hCG-α and the absence of immunological cross-reactivity of eCG-β with hCG-β. Also, only in the case of eCG is there a notable decrease in biological activity after dissociation and subsequent reassociation of subunits [3]. Furthermore, desialylated eCG shows an increased ability to stimulate Leydig cell testosterone production [4], whereas desialylation of hCG results in a decreased stimulatory activity [14, 15]. Since for hCG it is known that the carbohydrate moiety is essential for the biological functioning of the hormone [16–24] it could well be that functional differences between eCG and hCG derive from differences in their carbohydrate structures. In this paper we report on the structural analysis of the *N*- and *O*-glycans of eCG.

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Abbreviations. PNGase-F, peptide-N^4-(*N*-acetyl-β-glucosaminyl)asparagine amidase F; Endo-F, endo-β-*N*-acetylglucosaminidase-F; eCG, equine chorionic gonadotropin; eCG-α, α subunit; eCG-β, β subunit; hCG, human chorionic gonadotropin; eLH, equine luteinizing hormone; Fuc, L-fucose; Neu5Ac, *N*-acetylneuraminic acid; Neu4,5Ac$_2$, *N*-acetyl-4-*O*-acetylneuraminic acid; R_x, relative (HP)LC retention volume of compound *x*.

Enzymes. Peptide-N^4-(*N*-acetyl-β-glucosaminyl)asparagine amidase F (EC 3.5.1.52); endo-β-*N*-acetylglucosaminidase F (EC 3.2.1.96).

MATERIALS AND METHODS

Enzyme

Peptide-N^4-(*N*-acetyl-β-glucosaminyl)asparagine amidase F (PNGase-F) from *Flavobacterium meningosepticum* was obtained from Boehringer Mannheim.

Purification of eCG and preparation of subunits

Partially purified eCG (50 mg, 1810 IU/mg, ovarian augmentation test), supplied by Diosynth (Oss, The Netherlands) contained several protein contaminants as demonstrated by

SDS/PAGE [25]. The eCG sample was purified by gel-permeation chromatography on a Sephacryl S-200 SF column (122 × 1.5 cm, Pharmacia) using 0.02 M NH_4OAc, adjusted to pH 6 with acetic acid, as eluent at a flow rate of 8 ml/h. For the preparation of subunits the eCG-containing fraction was lyophilized and incubated for 1 h at 37 °C in 8 M urea, adjusted to pH 6.0 with 0.01 M Na_2HPO_4/NaH_2PO_4, containing 0.9% (by mass) NaCl. Subsequently, the subunits were separated by gel-permeation chromatography on a Sephacryl S-200 SF column (121 × 1.0 cm) in the same urea buffer, adjusted to pH 7.4 at a flow rate of 9 ml/h.

Liberation of the N- and O-linked carbohydrate chains

The N-linked carbohydrate chains were released from the protein moiety essentially as described earlier [26], the major difference being that in this case non-immobilized PNGase-F was used. Briefly, purified eCG-β (6.4 mg) and eCG-α (2.6 mg) were each dissolved in 0.5 ml 50 mM Tris, adjusted with HCl to pH 7.2, containing 10 mM EDTA, 1% (mass/vol.) SDS, 1% (by vol.) 2-mercaptoethanol, and then kept at 40 °C for 1 h. Subsequently, 10 µl NP-40 was added to both samples and after mixing thoroughly, 3 U PNGase-F was introduced in each tube. Incubation was carried out at ambient temperature for 48 h in an end-over-end mixer. Completeness of liberation of the N-linked chains was checked by SDS/PAGE. After desalting, using a Bio-Gel P-2 column (18 × 1 cm, 200–400 mesh, Bio-Rad) and lyophilization, the sample was fractionated on a Bio-Gel P-6 column (50 × 1.9 cm, 200–400 mesh, Bio-Rad) using 0.05 M NH_4HCO_3, adjusted to pH 7 with 1 M HCl, as eluent. Carbohydrate-positive material (orcinol/H_2SO_4 spot test) was pooled and lyophilized. In case of eCG-β, the *N*-deglycosylated *O*-glycoprotein-SDS complex was treated with alkaline borohydride as described [26] to release the O-linked carbohydrate chains. The β-elimination reaction products were desalted on Bio-Gel P-2 and lyophilized. A second batch of purified eCG-β (10.4 mg) and eCG-α (4.2 mg) was worked up in the same way.

FPLC fractionation of the N- and O-linked carbohydrate chains

Fractionation of the enzymatically and chemically released carbohydrate chains was carried out on a Mono Q HR 5/5 anion-exchange column (Pharmacia FPLC system) according to charge as described [26]. The fractions, monitored at 214 nm, were collected, desalted on Bio-Gel P-2 and lyophilized. For quantification of the oligosaccharide material present in the FPLC fractions, the absorbance at 214 nm for the sugar acyl groups was calibrated using known amounts of Neu5Ac, GlcNAc and GlcNAcβ1-4GlcNAc. It was demonstrated that the absorbance is linear in the range 0–100 nmol Neu5Ac (0–200 nmol C = O groups), and that the molar absorbances of Neu5Ac:GlcNAc:GlcNAcβ1-4GlcNAc at 214 nm amount to 2:1:2 in this range.

HPLC subfractionation of carbohydrate chains

Subfractionation of the carbohydrate-containing FPLC fractions was carried out with a Kratos Spectroflow 400 HPLC system using a Lichrosorb-NH_2 10-µm column (25 × 0.46 cm, Chrompack). Samples were dissolved in 0.05 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.). Runs were monitored at 205 nm with a Spectroflow 783 programmable absorbance detector and peaks were integrated by a Spectra Physics SP 4290 integrator. The corresponding HPLC fractions from the two subfractionations were pooled and desalted on Bio-Gel P-2.

Table 1. *Molar carbohydrate composition of eCG*
A, B, C and D: data according to [3], [4], [2] and this study, respectively. Man was taken as 3.0 residues

Monosaccharide	A	B	C	D
Fuc	1.0	0.3	0.6	0.7
Man	3.0	3.0	3.0	3.0
Gal	15.1	20.1	10.3	13.5
GlcNAc	9.8	15.9	12.3	13.8[a]
GalNAc	3.5	4.8	3.2	5.2
Neu5Ac	11.0	9.1	6.7	8.3

[a] Corrected for the amount of Asn-linked *N*-acetylglucosamine that is not cleaved under the conditions of methanolysis.

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 Chemistry, 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) [27].

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 i.d., Chrompack) using a Varian Aerograph 3700 gas chromatograph. The trimethylsilylated methyl glycosides were prepared by methanolysis, *N*-(re)acetylation and trimethylsilylation [28].

RESULTS

Gel-permeation chromatography on Sephacryl S-200 SF of the eCG sample (1810 IU/mg) gives rise to partially overlapping peaks in the high-molecular-mass range (R_f 1.2–1.5, Fig. 1). Fraction 1 ($R_1 = 1.28 \times R_{d.b.}$, where d.b. = dextran blue) is the eCG-containing fraction [3] as checked by SDS/PAGE followed by Western blotting using polyclonal anti-eCG-β antibodies. Approximately 100 µg of the material is desalted on Bio-Gel P-6 for sugar analysis (Table 1D) and, comparison with sugar analysis data of earlier studies (Table 1, A–C), indicates that, although the general pattern remains, there is no consensus as to the exact quantitative composition. After treatment of the remaining material with 8 M urea in phosphate buffer, pH 6.0, gel-permeation chromatography on Sephacryl S-200 SF yields three fractions (Fig. 2). Fraction 2 ($R_2 = 1.32 \times R_{d.b.}$) and fraction 3 ($R_3 = 1.65 \times R_{d.b.}$) correspond to the eCG-β (6.4 mg) and eCG-α

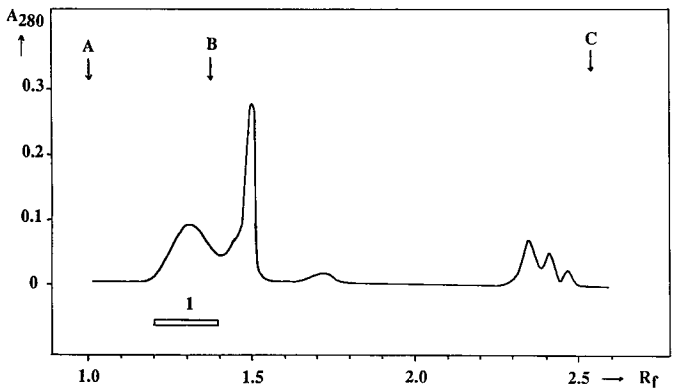


Fig. 1. *Gel-filtration pattern of partially purified eCG (1810 IU/mg) on a Sephacryl S-200 SF column (122 × 1.5 cm).* The elution was carried out with 0.02 M NH_4OAc, pH 6, at a flow rate of 8 ml/h. The eCG-containing fraction 1 was pooled as indicated. Arrows indicate the elution volumes of reference compounds: A, dextran blue ($R_{d.b.}$ = 1.00); B, human serotransferrin; C, *p*-nitrophenyl α-D-mannopyranoside

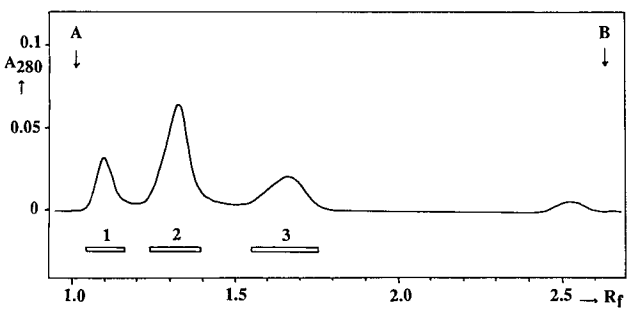


Fig. 2. *Separation of eCG subunits on a Sephacryl S-200 SF column (121 × 1.0 cm).* The elution was carried out with 8 M urea, adjusted to pH 7.4 with 0.01 M Na_2HPO_4/NaH_2PO_4, containing 0.9% (by mass) NaCl, at a flow rate of 9 ml/h. Fractions were pooled as indicated. Arrows indicate the elution volumes of reference compounds: A, dextran blue ($R_{d.b.}$ = 1.00); B, *p*-nitrophenyl α-D-mannopyranoside

(2.6 mg) subunits, respectively, as checked by SDS/PAGE followed by Western blotting using polyclonal anti-eCG-β and anti-eCG-α antibodies. The peak eluting slightly after the void volume of the column (fraction 1, $R_1 = 1.08 \times R_{d.b.}$) may represent an aggregation product of eCG [2] but was not investigated further.

N-linked carbohydrate chains

After treatment of SDS/2-mercaptoethanol-denatured eCG-β (6.4 mg) with PNGase-F, the desalted digest was fractionated on Bio-Gel P-6. The void volume peak containing the *N*-deglycosylated *O*-glycoprotein was collected for alkaline borohydride treatment (see below). The remaining pool of carbohydrate-positive material was chromatographed over Mono Q and collected in four main fractions, denoted N1β, N2β, N3β and N4β (Fig. 3). Fraction N4β, which derives from traces of SDS and SDS-protein complex, was combined with the *O*-glycoprotein-containing Bio-Gel P-6 fraction. The retention volumes of N1β (75 nmol C = O groups), N2β (760 nmol C = O groups) and N3β (430 nmol C = O groups) are identical to those obtained for mono-, di- and trisialo-oligosaccharides, respectively, isolated from human sero-

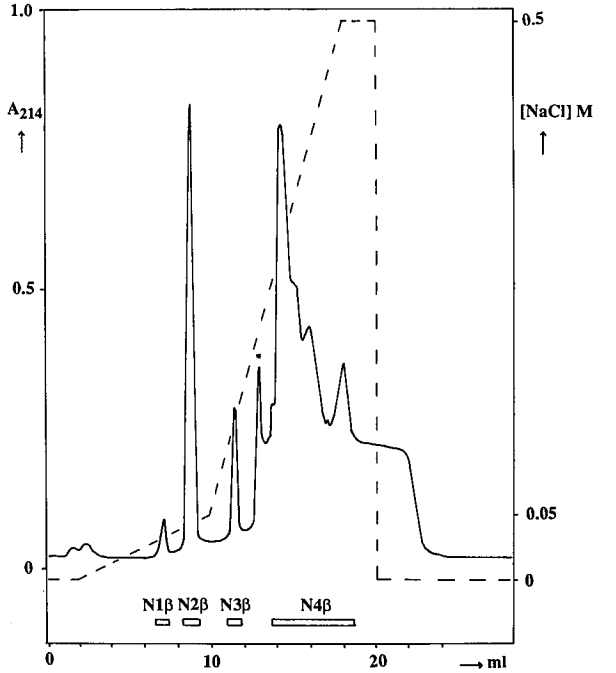


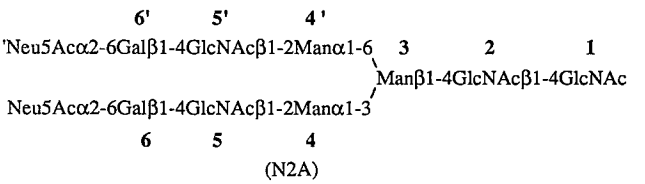
Fig. 3. *Fractionation pattern of the PNGase-F digestion products derived from eCG-β on a FPLC HR 5/5 Mono Q column.* The Bio-Gel P-6 carbohydrate fraction from PNGase-F-treated eCG-β was lyophilized and dissolved in 0.7 ml H_2O (HPLC quality). The column was eluted with a linear concentration gradient (----) 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 0.5 ml. Fractions were collected as indicated. * Signal stemming from traces of remaining Tris/HCl buffer

transferrin [29] and hCG [31]. PNGase-F-treated eCG-α (2.6 mg) shows a similar Mono Q pattern as eCG-β, except that N3α gives a double peak (Fig. 4). The peak areas of N1α, N2α and N3α correspond to 50, 400 and 190 nmol C = O groups, respectively. Fraction N4α does not contain carbohydrate material. The fractions N1β – N3β and N1α – N3α were analyzed by 500-MHz ^{1}H-NMR spectroscopy.

Fraction N2β: disialo-diantennary N-*acetyllactosamine type of oligosaccharides from eCG-β*

^{1}H-NMR spectroscopy of N2β demonstrates a high heterogeneity. Therefore, it was fractionated by HPLC on Lichrosorb-NH_2, giving rise to three major peaks, denoted N2β2, N2β4 and N2β6 and five minor peaks, denoted N2β1, N2β3, N2β5, N2β7 and N2β8 (Fig. 5).

Fraction N2β6 has the same HPLC retention volume ($R_{N2\beta6}$ = 1.00) as Neu5Acα2-6Galβ1-4GlcNAcβ1-2Manα1-6(Neu5Acα2 - 6Galβ1 - 4GlcNAcβ1 - 2Manα1 - 3)Manβ1 - 4GlcNAcβ1-4GlcNAc (HST), derived from human serotransferrin. ^{1}H-NMR analysis of this fraction (Table 2) and comparison with the ^{1}H-NMR data of reference oligosaccharide HST [30], confirm that fraction N2β6 represents compound N2A.



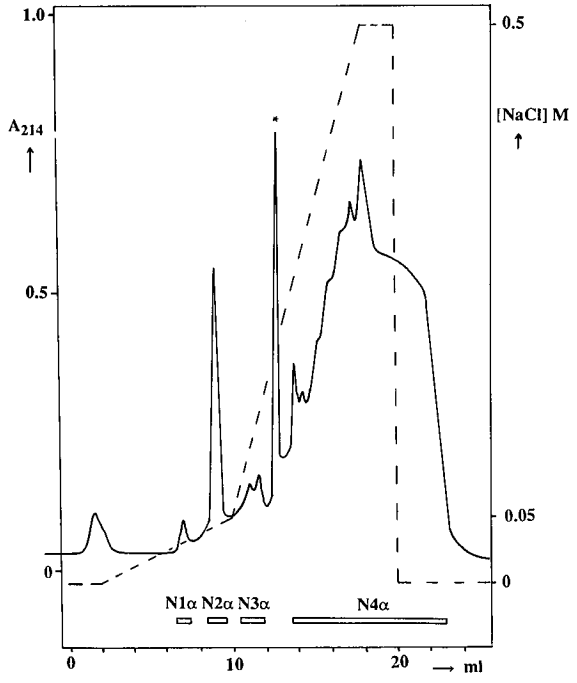


Fig. 4. *Fractionation pattern of the PNGase-F digestion products derived from eCG-α on a FPLC HR 5/5 Mono Q column.* For further details, see Fig. 3

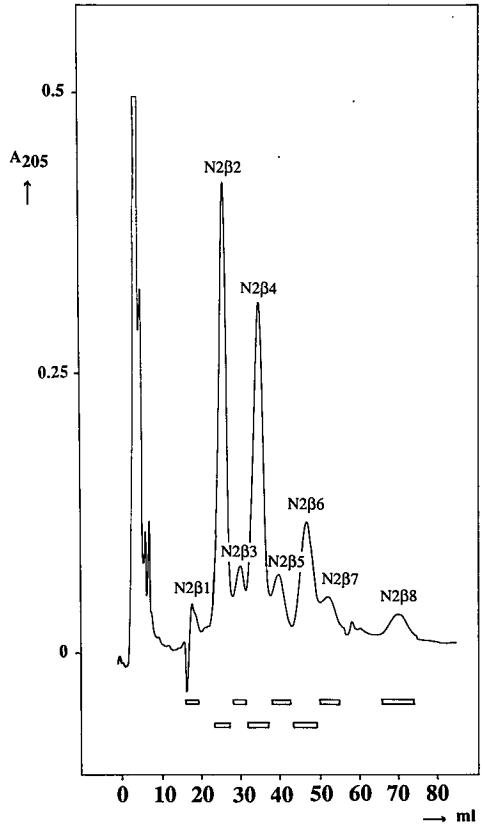
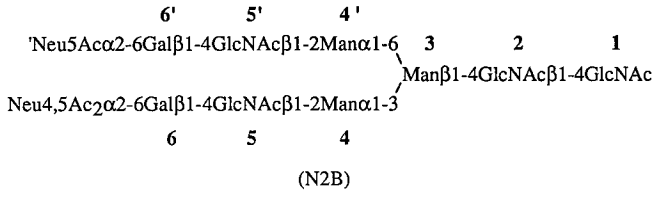
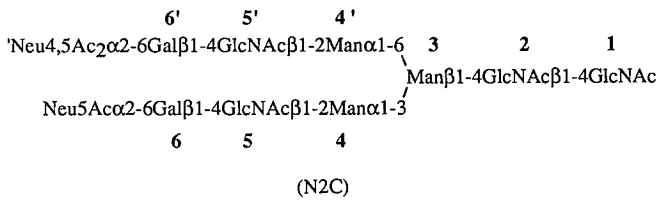


Fig. 5. *Fractionation pattern of the eCG-β FPLC fraction N2β on a HPLC Lichrosorb-NH_2 10-μm column (25 × 0.46 cm).* The FPLC fraction was lyophilized, desalted and dissolved in 0.05 ml 30 mM K_2HPO_4/KH_2PO_4, pH 7.0/acetonitrile (35:65, by vol.). The column was eluted isocratically with the same buffer at a flow rate of 120 ml/h at 25.0 °C. Injection volume 0.025 ml. Fractions were collected as indicated

Fraction N2β4 has the same HPLC retention volume ($R_{N2\beta4} = 0.73 \times R_{N2\beta6}$) as reference compounds Neu4,5Ac_2α2-6Galβ1-4GlcNAcβ1-2Manα1-6(Neu5Acα2-6Galβ1-4GlcNAcβ1-2Manα1-3)Manβ1-4GlcNAcβ1-4GlcNAc (eF.N2.2B) and Neu5Acα2-6Galβ1-4GlcNAcβ1-2Manα1-6(Neu4,5Ac_2α2-6Galβ1-4GlcNAcβ1-2Manα1-3)Manβ1-4GlcNAcβ1-4GlcNAc (eF.N2.2A) derived from equine fibrinogen [30]. As was reported earlier [30], *O*-acetylation at C-4 of a Neu5Ac residue as part of a disialodiantennary oligosaccharide results in a reduction of the retention volume by a factor of 0.73, regardless of whether the Neu4,5Ac_2 residue occurs on the Manα1-3 or the Manα1-6 branch. ^{1}H-NMR analysis of fraction N2β4 and comparison with the ^{1}H-NMR data of oligosaccharides eF.N2.2A and eF.N2.2B [30], shows it to consist of a mixture of equal amounts of N2B and N2C (Table 2).



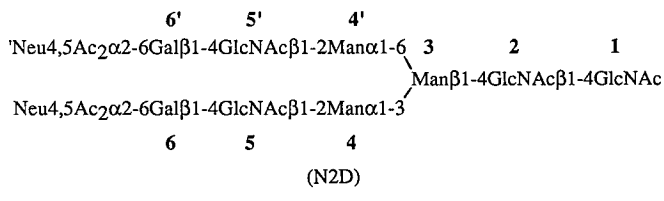
(N2B)



(N2C)

This conclusion is corroborated by the finding that, on HPLC, fraction N2β4 gives rise to a single peak having a retention volume between those of a diantennary compound in which both branches terminate in Neu5Ac (N2A, see above) and a diantennary compound in which both branches terminate in Neu4,5Ac_2 (N2D, see below).

Fraction N2β2 has the same HPLC retention volume ($R_{N2\beta2} = 0.54 \times R_{N2\beta6}$) as Neu4,5$Ac_2$α2-6Galβ1-4GlcNAcβ1-2Manα1-6(Neu4,5Ac_2α2-6Galβ1-4GlcNAcβ1-2Manα1-3)Manβ1-4GlcNAcβ1-4GlcNAc (eF.N2.1) derived from equine fibrinogen [30]. ^{1}H-NMR analysis of fraction N2β2 and comparison with the ^{1}H-NMR data for eF.N2.1 [30] confirm the presence of compound N2D as the single constituent (Table 2).



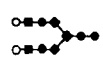
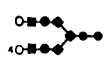
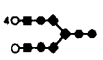
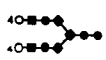
(N2D)

The reduction of the HPLC retention volume by a factor of 0.54 is in accordance with the presence of two Neu4,5Ac_2 residues, α2-6-linked to Gal-**6** and Gal-**6′**, respectively, each resulting in a reduction of the retention volume by a factor of 0.73. In Table 3 the HPLC retention volumes and the relative molar amounts of compounds N2A – N2D are compiled.

^{1}H-NMR spectroscopic analysis of the oligosaccharides present in the minor HPLC peaks is not feasible because of their low amounts. However, inspection of the ^{1}H-NMR spectrum of the total fraction N2β reveals the presence of

Table 2. *^{1}H-chemical shifts of structural-reporter-group protons of the constituent monosaccharides for the oligosaccharides N2A, N2B, N2C and N2D derived from eCG-β*

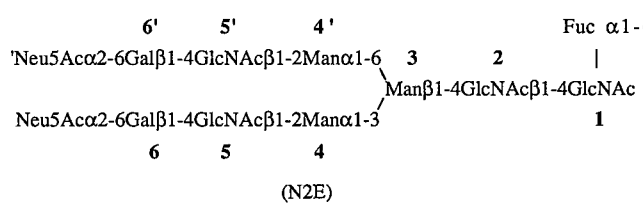
Chemical shifts are given at 300 K downfield from internal sodium 4,4-dimethyl-4-silapentane-1-sulphonate in ^2H_2O, but were actually measured by reference to internal acetone ($\delta = 2.225$ ppm in ^2H_2O at 300 K). Compounds are represented by short-hand symbolic notation [27]: ●, GlcNAc; ◆, Man; ■, Gal; ○, Neu5Acα2-6; 4O, Neu4,5Ac$_2$α2-6. For numbering of the monosaccharide residues, see text

Reporter group	Residue	Chemical shift in			
		N2A 	N2B 	N2C 	N2D 
		ppm			
H-1	GlcNAc-**1**α	5.190	5.190	5.190	5.190
	β	4.697	4.697	4.697	4.697
	GlcNAc-**2**α	4.614	4.615	4.615	4.616
	β	4.606	4.606	4.606	4.609
	Man-**4**	5.133	5.138	5.135	5.144
	Man-**4′**	4.947	4.949	4.953	4.958
	GlcNAc-**5**	4.606	4.606	4.606	4.609
	GlcNAc-**5′**	4.606	4.606	4.606	4.609
	Gal-**6**	4.444	4.440	4.443	4.440
	Gal-**6′**	4.448	4.448	4.443	4.442
H-2	Man-**3**	4.257	4.259	4.259	4.262
	Man-**4**	4.198	4.202	4.202	4.204
	Man-**4′**	4.117	4.120	4.120	4.124
H-3a	Neu5Ac	1.718	–	1.718	–
	Neu5Ac′	1.718	1.718	–	–
	Neu4,5Ac$_2$	–	1.850	–	1.850
	Neu4,5Ac$_2$′	–	–	1.850	1.850
H-3e	Neu5Ac	2.671	–	2.667	–
	Neu5Ac′	2.671	2.672	–	–
	Neu4,5Ac$_2$	–	2.672	–	2.678
	Neu4,5Ac$_2$′	–	–	2.684	2.682
H-4	Neu4,5Ac$_2$	–	4.900	–	4.903
	Neu4,5Ac$_2$′	–	–	4.900	4.903
NAc	GlcNAc-**1**	2.037	2.037	2.037	2.038
	GlcNAc-**2**	2.083	2.084	2.084	2.085
	GlcNAc-**5**	2.068	2.110	2.069	2.110
	GlcNAc-**5′**	2.065	2.066	2.103	2.104
	Neu5Ac	2.030	–	2.030	–
	Neu5Ac′	2.030	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

oligosaccharides with the following characteristics: (a) missing the GlcNAc-**1** residue, (b) bearing Fuc in α1-6 linkage to GlcNAc-**1**, and (c) having α2-3-linked Neu5Ac at Gal-**6′**.

The glycans having GlcNAc-**2** in reducing position are indicated by the GlcNAc-**2** NAc singlet at $\delta = 2.059$ ppm and the GlcNAc-**2** H-1α doublet at $\delta = 5.217$ ppm [32]. This finding demonstrates the presence of endo-F activity in the PNGase-F enzyme preparation used. The relative intensities of these signals in the ^{1}H-NMR spectrum of fraction N2β indicate that approximately 10% of the total amount of oligosaccharides are missing the GlcNAc-**1** residue.

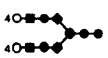
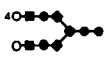
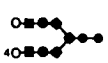
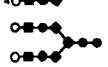
The presence of glycan(s) bearing Fuc in α1-6 linkage to GlcNAc-**1** (< 5% of the total amount of oligosaccharides) is indicated in the ^{1}H-NMR spectrum of fraction N2β by the characteristic signals at $\delta = 1.210$ ppm (Fuc-CH$_3$, α-GlcNAc-**1**), $\delta = 1.221$ ppm (Fuc-CH$_3$, β-GlcNAc-**1**) and $\delta = 2.098$ ppm (NAc of GlcNAc-**2**) [26]. The Fuc H-1 signal at $\delta = 4.893$ ppm is superimposed on the Neu4,5Ac$_2$ H-4 octet at $\delta = 4.904$ ppm. Therefore, and based on the knowledge that α1-6 fucosylation of GlcNAc-**1** results in an increase of the retention volume of the oligosaccharide by a factor 1.11 [31], it is suggested that fraction N2β7 ($R_{N2\beta7} = 1.11 \times R_{N2\beta6}$) represents:



(N2E)

The occurrence of a small amount (10% of total Neu5Ac) of α2-3-linked Neu5Ac at Gal-**6′** is shown in the ^{1}H-NMR spectrum of the composite fraction N2β by the characteristic

Table 3. *HPLC retention volumes (V_e) and relative molar amounts of the major disialo diantennary* N-*acetyllactosamine type of oligosaccharides derived from eCG-β*
Oligosaccharides were released from the glycoprotein by treatment with PNGase-F and fractionated according to charge by FPLC on Mono Q. The FPLC disialo oligosaccharide fraction was subfractionated by HPLC on Lichrosorb-NH_2. The relative molar amounts are calculated from the HPLC peak areas at 205 nm (corrected for the number of C=O groups)

Structure	Name	V_e	Relative amount
		ml	%
	N2D	24.02	46
	N2C	32.78	39
	N2B		
	N2A	44.68	15

H-3e and H-3a chemical shift values [27] and the signal at δ = 2.043 ppm (NAc of GlcNAc-**5′**). Signals indicating Neu5Ac in α2-3 linkage to Gal-**6** are absent.

Finally, it can be stated that the overall molar ratio Neu4,5Ac_2/Neu5Ac is 3:2, as concluded from the relative intensities of the NAc signals in the ^{1}H-NMR spectrum of fraction N2β of Neu4,5Ac_2 (δ = 1.964 ppm) and Neu5Ac (δ = 2.030 ppm).

Fraction N1β: monosialo N-*acetyllactosamine type of oligosaccharides from eCG-β*

^{1}H-NMR spectroscopy of fraction N1β reveals the presence of diantennary compounds carrying Neu5Ac residues either in α2-6 linkage or in α2-3 linkage in a molar ratio of approximately 1:2. Part of the α2-6-linked sialic acid residues are *O*-acetylated at C-4 (Neu4,5Ac_2 NAc and OAc at δ = 1.964 ppm and δ = 2.078 ppm, respectively), affording a molar ratio Neu4,5Ac_2/Neu5Ac of 1:3. Approximately 60% of the GlcNAc-**1** residues carry α1-6-linked Fuc, which is evident from the relative intensities of the GlcNAc-**2** NAc signals at δ = 2.084 ppm (non-fucosylated compounds) and δ = 2.098 ppm (fucosylated compounds). Upon HPLC, fraction N1β gives rise to eight peaks, demonstrating the large heterogeneity of the monosialo eCG-β oligosaccharides. Some of the peaks show surprisingly high retention volumes, indicating higher branched and/or poly(*N*-acetyllactosamine) type of carbohydrates. However, due to their low amounts these fractions could not be analyzed by ^{1}H-NMR spectroscopy.

Fraction N3β: trisialo N-*acetyllactosamine type of oligosaccharides from eCG-β*

HPLC of fraction N3β yields eight peaks. The structures of the oligosaccharides present in the individual fractions could not be established by ^{1}H-NMR spectroscopy due to their low amounts. Therefore, the general features of these structures were studied by ^{1}H-NMR spectroscopy of the composite fraction N3β with the following results.

a) N3β consists of a mixture of trisialo tri-antennary (H-2 signals of Man-**3**, Man-**4** and Man-**4′** at $\delta \approx 4.21$ ppm, $\delta \approx 4.22$ ppm and $\delta \approx 4.11$ ppm, respectively [27]) and trisialo tri′-antennary (H-2 signals of Man-**3**, Man-**4** and Man-**4′** at δ = 4.246 ppm, $\delta \approx 4.20$ ppm and $\delta \approx 4.10$ ppm, respectively [27]) compounds in a molar ratio of about 2:1.

b) Part of the terminal sialic acid residues occur in α2-6 linkage. Approximately 50% of these residues occur as Neu5Ac (H-3a at δ = 1.719 ppm, H-3e at δ = 2.678 ppm and NAc at δ = 2.031 ppm [27]). The remaining part of the α2-6-linked Neu5Ac residues are *O*-acetylated at C-4 (H-3a at δ = 1.852 ppm, H-3e at δ = 2.678 ppm, H-4 at δ = 4.906 ppm, NAc at δ = 1.964 ppm and OAc at δ = 2.078 ppm [30]).

c) Part of the sialic acid residues occur in α2-3 linkage in non-reducing terminal position. Approximately 50% of these residues occur as Neu5Ac (H-3a at δ = 1.803 ppm, H-3e at δ = 2.757 ppm and NAc at δ = 2.031 ppm [27]). The remaining part of the α2-3-bound Neu5Ac residues are 4-*O*-acetylated (H-3a at δ = 1.930 ppm, H-3e at δ = 2.770 ppm, NAc at δ = 1.964 ppm and OAc at δ = 2.078 ppm [33]).

d) About 20% of the GlcNAc-**1** residues bear Fuc in α1-6 linkage (CH_3 signals of Fuc at δ = 1.212 ppm (α-GlcNAc-**1**) and at δ = 1.223 ppm (β-GlcNAc-**1**) and an NAc signal of GlcNAc-**2** at δ = 2.097 ppm [26]).

It has to be noted that the occurrence of α2-3-linked Neu4,5Ac_2 seems to be restricted to the tri′-/tri-antennary carbohydrates of eCG since it was not detected in the monosialo and disialo diantennary oligosaccharides.

Fraction N2α: disialo N-*acetyllactosamine type of oligosaccharides from eCG-α*

HPLC on Lichrosorb-NH_2 of the FPLC fractions N1α, N2α and N3α gave rise to more complex peak patterns than those observed for the corresponding FPLC fractions of the β subunit, and only a preliminary characterization of the disialo glycans from composite FPLC fraction N2α, based on ^{1}H-NMR data, could be achieved. (a) The oligosaccharides are diantennary in structure. (b) Approximately 60% of the Manα1-3 and Manα1-6 branches carry α2-6-linked Neu5Ac in non-reducing terminal position. (c) Approximately 20% of the Manα1-3 and Manα1-6 branches carry α2-3-linked Neu5Ac in non-reducing terminal position. (d) The remaining 20% of the Manα1-3 and Manα1-6 branches terminate in α2-6-linked Neu4,5Ac_2. (e) About 90% of the GlcNAc-**1** residues have α-Fuc linked at C-6. (f) A substantial amount (about 30%) of oligosaccharides bear GlcNAc-**2** in a reducing position, indicating the presence of endo-F activity in the PNGase-F batch used to deglycosylate the α subunit of eCG.

The relative amounts of the structural elements mentioned above are based on the relative intensities of the corresponding ^{1}H-NMR signals. Comparison with the data for the disialo diantennary oligosaccharides stemming from eCG-β (fraction N2β) indicates that the disialo glycans of eCG-α show less *O*-acetylation of the α2-6-linked sialic acid residues, have a larger part of the sialic acid residues in α2-3 linkage and possess a much higher degree of GlcNAc-**1** α1-6 fucosylation.

O-linked carbohydrate chains

After removal of the Asn-linked oligosaccharides from the β subunit, the *O*-glycosidically linked chains are liberated by alkaline borohydride treatment and separated as oligosaccharide-alditols on Mono Q (Fig. 6). As is evident from quantitative sugar analysis (data not shown), the neutral fraction O1 contains in addition to non-carbohydrate material traces of Gal, GalNAc, GlcNAc and Neu5Ac suggesting the pres-

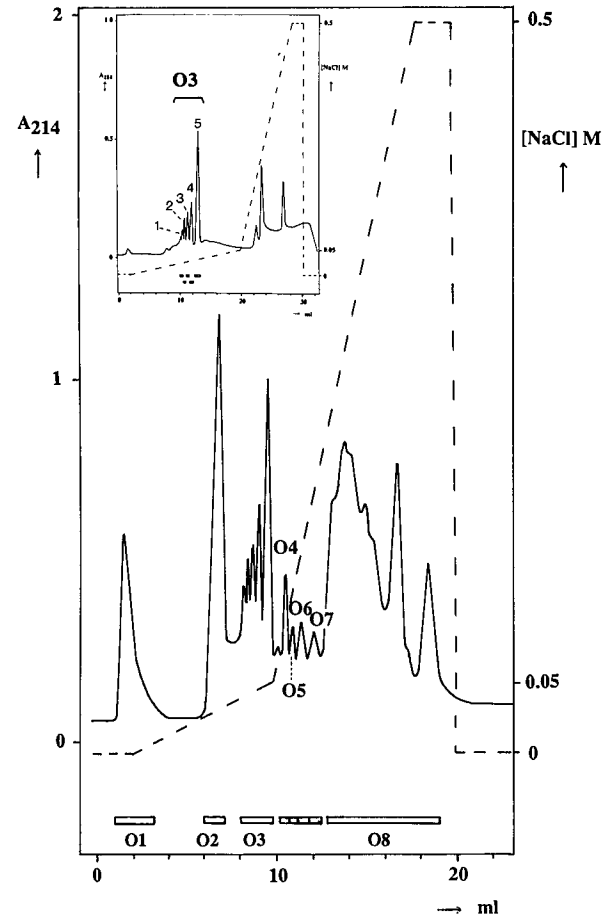


Fig. 6. *Fractionation pattern of the β-elimination products derived from N-deglycosylated eCG-β on a FPLC HR 5/5 Mono Q column.* Conditions as in Fig. 3. The FPLC fraction O3 was desalted, lyophilized and dissolved in 0.7 ml H_2O (HPLC quality) and rechromatographed on Mono Q (insert). In this case the column was eluted with a linear concentration gradient (----) of 0–50 mM NaCl in 18 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 0.5 ml. Fractions were collected as indicated

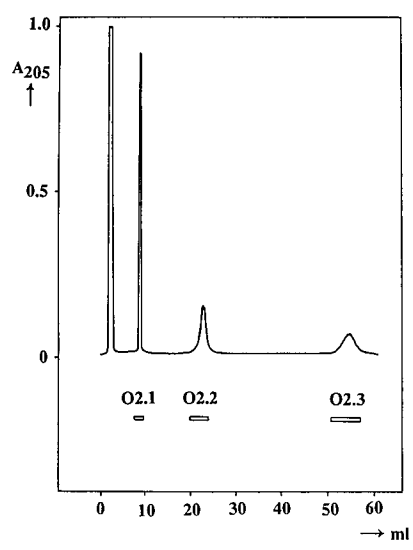


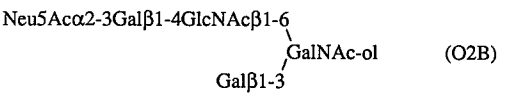
Fig. 7. *Fractionation pattern of the eCG-β FPLC fraction O2 on a HPLC Lichrosorb-NH_2 10-μm column (25 × 0.46 cm).* The column was eluted isocratically with 30 mM K_2HPO_4/KH_2PO_4, pH 7.0/acetonitrile (30:70, by vol.). For further details, see Fig. 5

ence of residual glycopeptide material. The elution volumes suggest that fraction O2 represents monosialo oligosaccharides, fractions O3 and O4 disialo, and fractions O5–O7 trisialo oligosaccharides. O8 does not contain carbohydrate material (sugar analysis). Based on the absorbances at 214 nm, fractions O2, O4, O5, O6 and O7 contain 1540, 85, 35, 40 and 30 nmol C = O groups, respectively. To accomplish better separation, fraction O3 was desalted and rechromatographed on Mono Q using a less steep gradient (insert Fig. 6). Five disialo oligosaccharide fractions were collected, denoted O3.1–O3.5. The absorbances at 214 nm demonstrate that these fractions contain 25, 55, 65, 140 and 450 nmol C = O groups, respectively.

HPLC subfractionation of O2 on Lichrosorb-NH_2 yielded three fractions, denoted O2.1, O2.2 and O2.3 (Fig. 7), of which the ratio of the absorbances at 205 nm is 1:0.2:0.1. ^{1}H-NMR spectroscopy demonstrates that fraction O2.1 contains O2A as the single constituent. The structural-reporter-group data match those of the same compound isolated from hCG (hCG.O1) [26] (Table 4).

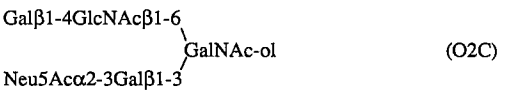
Neu5Acα2-3Galβ1-3GalNAc-ol (O2A)

^{1}H-NMR analysis of O2.2 shows the presence of the following compounds



(O2B)

and



(O2C)

The presence of exclusively α2-3-linked Neu5Ac is evident from the H-3a signal at δ = 1.798 ppm. The NAc singlet of GalNAc-ol of both compounds is visible at δ = 2.065 ppm (Table 4). The occurrence of O2B is clear from the set of structural-reporter-group signals at δ = 2.758 ppm (H-3e of Neu5Ac), δ = 4.393 ppm, δ = 4.058 ppm and δ = 4.281 ppm (H-2, H-3 and H-5 of GalNAc-ol), δ = 4.465 ppm (H-1 of Gal^3), δ = 2.061 ppm and δ = 4.552 ppm (NAc and H-1 of $GlcNAc^6$), and δ = 4.548 ppm and δ = 4.114 ppm (H-1 and H-3 of Gal^4) (cf. b-5 [34]). The presence of O2C is evidenced by a second set of structural-reporter-group signals, namely, at δ = 2.779 ppm (H-3e of Neu5Ac), δ = 4.389 ppm, δ = 4.074 ppm and δ = 4.269 ppm (H-2, H-3 and H-5 of GalNAc-ol), δ = 4.533 ppm and δ = 4.114 ppm (H-1 and H-3 of Gal^3), δ = 4.563 ppm (H-1 of $GlcNAc^6$) and δ = 4.472 ppm (H-1 of Gal^4) (cf. b-4 [34]). The NAc singlets of GlcNAc and GalNAc-ol coincide at δ = 2.065 ppm. From the intensities of the two sets of signals, belonging to O2B and O2C, respectively, it can be inferred that the molar ratio of O2B/O2C is 3:2.

Based on its chromatographic behaviour, it can be assumed that O2.3 represents a relatively large oligosaccharide (HPLC retention volume) having one sialic acid residue (FPLC retention volume). The ^{1}H-NMR spectrum of fraction O2.3, which can not be interpreted entirely thus far, supplies indications for the presence of (a) poly(*N*-acetyllactosamine) type of oligosaccharide(s). The occurrence of this type of oligosaccharide(s) in eCG was suggested earlier by Bahl et al. [8–10].

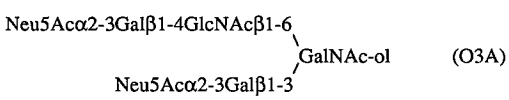
^{1}H-NMR spectroscopy of fraction O3.5 and comparison with the ^{1}H-NMR data of reference compound hCG.O2 [26]

Table 4. *^{1}H-chemical shifts of the structural-reporter-group protons of the constituent monosaccharides for the oligosaccharide-alditols O2A, O2B, O2C, O3A and O4 obtained from eCG-β*

A superscript at the name of a sugar residue indicates to which position of the adjacent monosaccharide it is glycosidically linked. A second superscript is used to discriminate between identically linked residues, by indicating the type of the next linkage in sequence. ◇–ol, GalNAc-ol; △, Neu5Acα2-3. For further details, see Table 2

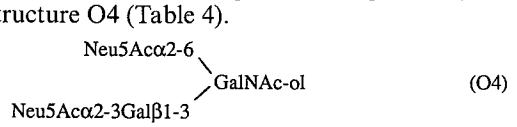
Residue	Reporter group	Chemical shift in				
		O2A 	O2B 	O2C 	O4 	O3A 
		ppm				
GalNAc-ol	H-2	4.388	4.393	4.389	4.378	4.389
	H-3	4.072	4.058	4.074	4.068	4.068
	H-4	3.495	3.477	n.d.	3.523	3.444
	H-5	4.187	4.281	4.269	4.238	4.266
	NAc	2.045	2.065	2.065	2.042	2.065
Gal^3	H-1	4.546	4.465	4.533	4.542	4.531
	H-3	4.121	n.d.	4.114	4.115	4.115
	H-4	3.929	3.895	3.935	3.927	n.d.
GlcNAc^6	H-1	–	4.552	4.563	–	4.552
	H-6	–	4.008	3.994	–	4.007
	NAc	–	2.061	2.065	–	2.065
Gal^4	H-1	–	4.548	4.472	–	4.547
	H-3	–	4.114	n.d.	–	4.115
	H-4	–	n.d.	n.d.	–	3.960
Neu5Ac^{3,3}	H-3a	1.799	–	1.798	1.799	1.800
	H-3e	2.772	–	2.779	2.775	2.780
	NAc	2.033	–	2.032	2.032	2.033
Neu5Ac^{3,4}	H-3a	–	1.798	–	–	1.800
	H-3e	–	2.758	–	–	2.756
	NAc	–	2.032	–	–	2.033
Neu5Ac^6	H-3a	–	–	–	1.692	–
	H-3e	–	–	–	2.725	–
	NAc	–	–	–	2.032	–

demonstrates that it contains oligosaccharide O3A as the single constituent (Table 4).



(O3A)

Likewise, fraction O4 is proven to represent (cf. hCG.O3 [26]) structure O4 (Table 4).



(O4)

From the chromatographic behaviour on Mono Q and the ^{1}H-NMR data, it can be deduced that O3.4–O3.1 and O7–O5 are disialo and trisialo mucin types of oligosaccharide, respectively, probably containing an increasing number of *N*-acetyllactosamine units (cf. [8–10]). These structures are still under investigation.

DISCUSSION

In this study PNGase-F was used to liberate the N-linked carbohydrate chains of eCG for structural analysis. As reported earlier [30], by virtue of the mild cleavage conditions, the use of PNGase-F allows determination of the occurrence of Neu4,5Ac$_2$ residues on the free and intact oligosaccharides. It is shown that eCG-β contains di- and tri'-/tri-antennary N-linked oligosaccharides. As in hCG-β, a disialo diantennary chain is the major glycan. However, whereas in hCG-β the molar ratio monosialo/disialo/trisialo is 19:74:7, in eCG-β this approximates to 9:64:27, indicating a shift towards higher sialylated (i.e. higher branched) carbohydrate chains (cf. [26, 31]). Furthermore, in eCG-β α2-6-linked Neu5Ac residues form the main sialic acid constituents (30%, 93% and 65% of total sialic acid in the mono-, di- and tri-sialo oligosaccharides, respectively), whereas in hCG only traces of α2-6-linked Neu5Ac residues have been found [26]. Finally, in hCG only Neu5Ac residues have been encountered, whereas in eCG-β the sialic acid constituents can be *O*-acetylated at C-4, namely 25%, 65% and 50% of the total number of sialic acid residues in the mono-, di- and tri-sialo glycans, respectively. Remarkably, the occurrence of Neu4,5Ac$_2$ in α2-3 linkage seems to be restricted to the tri'-/tri-antennary glycans.

Preliminary characterization of the disialo carbohydrate chains of eCG-α reveals that here approximately 80% of the sialic acid residues occur in α2-6 linkage (vs 20% in α2-3 linkage), and a much lower degree of *O*-acetylation, namely 20%.

The degree of fucosylation of GlcNAc-**1** in the N-linked mono-, di- and tri'-/tri-antennary oligosaccharides of eCG-β was found to be $\approx 60\%$, $< 5\%$ and $\approx 20\%$, respectively.

However, it should be noted that the values determined for the diantennary glycans may be too low in view of the partial degradation (affecting $\approx 10\%$ of total oligosaccharides) due to the presence of endo-F activity in the PNGase-F preparation used for *N*-deglycosylation.

Based on the amount of C = O groups (reflected by the absorbances at 214 nm) in the Mono Q fractions N1β – N3β and the structures determined for the major part of the oligosaccharides present in these fractions, the molar amounts can be calculated to be 13 nmol monosialo diantennary, 95 nmol disialo diantennary and 39 nmol trisialo tri'-/triantennary carbohydrate chains. Starting with 6.4 mg eCG-β this means that the β subunit contains approximately 6% (by mass) N-linked carbohydrate chains, or approximately one N-linked chain per subunit. Thus only one glycosylation site for N-linked chains seems to occur, showing however, a remarkably structural heterogeneity of the glycan attached to it (cf. [8 – 10]).

Establishment of the native pattern of base-labile functional groups (e.g. the 4-*O*-acetyl group of sialic acid) in the O-linked glycans presents problems since no suitable enzyme is available for the release of these carbohydrate chains from the protein backbone. Chemical liberation procedures, such as alkaline borohydride treatment, lead to the concomitant loss of these functional groups, whereas preparation of *O*-glycopeptides gives rise to separation problems, impeding preparation of pure fractions. In this study *O*-glycosidic oligosaccharides were released by alkaline borohydride treatment and consequently no statements can be made on the possible occurrence of 4-*O*-acetylated sialic acids in these chains.

The majority of the *O*-glycosidically linked chains, which were detected only on eCG-β, is accounted for by the trisaccharide Neu5Acα2-3Galβ1-3GalNAc-ol (O2A, 400 nmol). The disialo hexasaccharide O3A (75 nmol) and tetrasaccharide O4 (17 nmol) and the monosialo derivatives O2B (36 nmol) and O2C (24 nmol) form minor constituents. In addition a small amount of larger O-linked oligosaccharides, being very heterogeneous in structure, presumably due to a varying number of *N*-acetyllactosamine units, is present. Thus at least 547 nmol of O-linked carbohydrate chains occur on eCG-β, meaning that it contains at least four *O*-glycosylation sites.

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