Glycoconjugate J (1987) 4: 129-144

A General Strategy for the Isolation of Carbohydrate Chains from *N*-,*O*-Glycoproteins and its Application to Human Chorionic Gonadotrophin

JAN B L DAMM[1], JOHANNIS P KAMERLING[1], GIJS W K VAN DEDEM[2] and JOHANNES F G VLIEGENTHART[1]

[1]*Department of Bio-Organic Chemistry, Utrecht University, P.O. Box 80.075, NL-3508 Utrecht, The Netherlands*
[2]*Diosynth B.V., P.O. Box 20, NL-5340 BH Oss, The Netherlands*

Received February 17, 1987.

Key words: PNGase-F, hCG, ^{1}H-NMR, FPLC

For the structural analysis of the carbohydrate chains of *N*-,*O*-glycoproteins a straightforward strategy was developed based on the cleavage of the *N*-linked chains with immobilized peptide-N^4-(*N*-acetyl-β-glucosaminyl)asparagine amidase-F (PNGase-F) from *Flavobacterium meningosepticum*, followed by alkaline borohydride treatment of the remaining *O*-glycoprotein material. This methodology was applied to the isolation of the Asn- and Ser-linked carbohydrate chains of human chorionic gonadotrophin. The structures of the isolated oligosaccharides were verified by 500-MHz ^{1}H-NMR spectroscopy. The Asn-linked sugar chains were shown to be: NeuAcα2-3Galβ1-4GlcNAcβ1-2Manα1-6[NeuAcα2-3Galβ1-4GlcNAcβ1-2Manα1-3]Man β1-4GlcNAcβ1-4[Fucα1-6]$_{0-1}$GlcNAc and Manα1-6[NeuAcα2-3Galβ1-4GlcNAcβ1-2Man α1-3]Manβ1-4GlcNAcβ1-4GlcNAc. Also some minor constituents occurred. The structures of the Ser-linked oligosaccharides were established in the form of their oligosaccharide-alditols as: NeuAcα2-3Galβ1-3[NeuAcα2-6]GalNAc, NeuAcα2-3Galβ 1-3GalNAc and NeuAcα2-3Galβ1-3[NeuAcα2-3Galβ1-4GlcNAcβ1-6]GalNAc.

In general it is not possible to determine the primary structure of a carbohydrate chain on an intact glycoprotein. Usually glycoproteins contain multiple sugar chains at definite Asn and/or Ser/Thr residues with different structures. Furthermore, microheterogeneity of the carbohydrate chains is a known phenomenon. Therefore, preparation of partial structures of the polypeptide backbone having a single glycosylation position or liberation of the carbohydrate moieties from the polypeptide backbone is a prerequisite. For this purpose several chemical methods are available.

Procedures which split off both *N*- and *O*-linked chains are trifluoracetolysis [1] and

---

Abbreviations: hCG, human chorionic gonadotrophin; hCG-α, α-subunit; hCG-β, β-subunit; EIA, enzyme immunoassay; PNGase-F, peptide-N^4-(*N*-acetyl-β-glucosaminyl)asparagine amidase-F (EC 3.5.1.52); SDS, sodium dodecyl sulphate; GalNAc, *N*-acetylgalactosamine; GlcNAc, *N*-acetylglucosamine; NeuAc, *N*-acetylneuraminic acid; Man, mannose; Gal, galactose; Fuc, fucose.

alkaline hydrolysis [2]. However, these methods give rise to partial degradation of the reducing end of the carbohydrate chains. Also proteolytic digestion of the polypeptide backbone [3], resulting in heterogeneous peptide moieties linked to the carbohydrate chain, has its limitations. Moreover, when neighbouring chains occur, the intervening peptide backbone may resist proteolysis. Chemical methods that are claimed to be specific for the release of Asn-linked and Ser/Thr-linked oligosaccharides are hydrazinolysis [4] and alkaline borohydride treatment (β-elimination reaction) [5], respectively. However, the hydrazinolysis conditions also give rise partly to cleavage of (GalNAc-)Ser/Thr linkages [4, 6], and on the other hand alkaline borohydride conditions split (GlcNAc-)Asn linkages to a certain extent [6, 7]. In addition, data are accumulating that hydrazinolysis also causes some further degradation/conversion of *N*-linked chains [8-11], introducing still more heterogeneity. Thus, when a glycoprotein contains both *N*- and *O*-linked oligosaccharides, difficulties may be encountered because mixtures of *N*- and *O*-carbohydrate chains (and their degradation products) evolve using either hydrazinolysis or β-elimination. Such mixtures present problems in structure elucidation since they are difficult to separate. To circumvent these problems, an alternative method using an enzymatic approach to release the *N*-glycosidic chains was studied.

The use of endo-β-*N*-acetylglucosaminidases, leading to the cleavage of the core *N,N'*-diacetylchitobiose, is limited because these enzymes are to some extent carbohydrate chain specific [12-14]. Recently, some peptide-N^4-(*N*-acetyl-β-glucosaminyl)asparagine amidases have become commercially available, which seem to be very promising for the analysis of *N*-linked carbohydrate chains. The enzymes have been shown to release oligomannose-, hybrid- and *N*-acetyllactosamine di-, tri- and tetra-antennary types of carbohydrate chains, which makes them generally applicable. Until now three sources of this enzyme have been reported: PNGase-F from *Flavobacterium meningosepticum* [13-19], PNGase-A from almond emulsin [20, 21] and PNGase-JB from Jack beans [22]. Small differences in substrate specificity may occur. For proteins in their native conformation, the susceptibility to these enzymes can vary. The rate of deglycosylation is greatly enhanced if the glycoprotein substrate is denatured prior to enzyme treatment. The minimum oligosaccharide chain that is released from the protein by PNGase-F, the enzyme used in this study, is the *N,N'*-diacetylchitobiosyl core unit [16]. In the case of a glycopeptide even a single *N*-acetylglucosamine residue linked to Asn can be liberated, provided that both the α-amino and carboxyl groups of the Asn-residue are in a peptide linkage.

In the framework of our studies concerning the analysis of carbohydrate chains of *N*-,*O*-glycoproteins, we developed a strategy based on the cleavage of the *N*-linked chains with immobilized PNGase-F, followed by alkaline borohydride treatment of the remaining purified *O*-glycoprotein material. The procedure was tested on human chorionic gonadotrophin, as will be reported here.

Materials and Methods

Enzymes

Peptide-N^4-(*N*-acetyl-β-glucosaminyl)asparagine amidase-F (PNGase-F) isolated from *Flavobacterium meninogsepticum* (EC. 3.5.1.52) was a gift from Dr. W. Hösel (Boehringer Mannheim). β-Galactosidase from Jack beans (EC. 3.2.1.23) was obtained from Sigma Chemical Co. (St. Louis, MO, USA).

Immobilisation of Enzymes

Immobilisation was carried out essentially according to the procedure recommended by Pharmacia. Lyophilized CNBr-activated Sepharose 4B (Pharmacia, Uppsala, Sweden), 1 g, was swollen in 10 ml 1 mM HCl for 20 min and washed with 200 ml 1 mM HCl on a sintered glass filter. Freezedried PNGase-F (15 U; 1 U will hydrolyze 1 nmol dansylglycopeptide from fetuin per min at 37°C, in 20 mM potassium phosphate buffer, pH 7.2, containing 50 mM EDTA) was dissolved in 5 ml 0.1 M $NaHCO_3/Na_2CO_3$, pH 8.3, containing 0.5 M NaCl and mixed with the gel in a stoppered vessel. β-Galactosidase (2 U; 1 U will hydrolyze 1 μmol *p*-nitrophenyl-β-D-galactopyranoside per min at 37°C in 0.1 M sodium phosphate/citrate buffer, pH 5.5) was added as a suspension in 0.2 ml $(NH_4)_2SO_4$, containing 25 mM sodium citrate, to 5.8 ml 0.1 M $NaHCO_3/Na_2CO_3$, pH 8.3, containing 0.5 M NaCl, and mixed with the gel. The mixture was rotated in an end-over-end mixer for 16 h at 4°C, after which the remaining reactive groups were blocked with 10 ml 1 M ethanolamine/HCl, pH 8.0. Finally, the product was washed with 0.1 M $NaHCO_3/Na_2CO_3$, pH 8.3, containing 0.5 M NaCl, followed by a wash with 0.1 M sodium acetate buffer, pH 4.0, containing 0.5 M NaCl. After repeating this procedure five times the product was suspended in the appropriate enzyme buffer.

Preparation of Subunits

Highly purified urinary hCG (10 900 I.E./mg) used in these studies, was provided by Diosynth, Oss, The Netherlands. Subunits were prepared by incubating hCG 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% NaCl. The gel permeation chromatography on a Sephacryl S-200 column (121 × 0.52 cm) was carried out in the same urea buffer adjusted to pH 7.5. Subunit preparations were checked for purity by SDS-polyacrylamide gel electrophoresis and EIA.

Liberation and Fractionation of the N- and O-Glycosidic Carbohydrate Chains

To liberate the *N*-linked carbohydrate chains, 10 mg hCG were dissolved in 0.5 ml 50 mM K_2HPO_4, adjusted with KOH to pH 8.5, containing 10 mM EDTA, 2% (w/v) SDS, 1% (v/v) mercaptoethanol, and incubated on a Sepharose 4B column (1 g dry material) with immobilized PNGase-F for 48 h at room temperature. The reaction products were eluted with 8 ml 50 mM K_2HPO_4, pH 8.5, and desalted on a Bio-Gel P-2 column (35 × 1 cm, Bio-Rad Labs, Richmond, CA, USA; 200-400 mesh). After lyophilization, the material was redissolved in 0.7 ml water (HPLC-quality) and fractionated on a Mono Q HR 5/5 column (Pharmacia FPLC-system) at a flow rate of 60 ml/h, using a linear concentration gradient from 0-50 mM NaCl in 8 ml water (HPLC-quality), followed by a steeper gradient from 50-500 mM NaCl in 8 ml water. The *N*-glycosidic carbohydrate fractions were collected for further analysis. The protein-SDS fraction containing the *O*-linked carbohydrate chains was lyophilized, redissolved in 1 ml 0.1 M NH_4Ac buffer, pH 5.5, and passed through a Bio-Gel P-6 column (27 × 0.55 cm, 200-400 mesh, Bio-Rad) in the same buffer. The void volume peak was collected, lyophilized and dissolved in 5 ml 0.1 M NaOH, containing 1 M $NaBH_4$. After 25 h at 39°C the solution was neutralized to pH 6 with 4 M acetic acid and lyophilized. The material was suspended in 1 ml water and after centrifugation (5 min, 10 000 × *g*) the supernatant was desalted on a Bio-Gel P-2 column. After lyophilization the residue was fractionated on Mono Q as described above. The *N*- and *O*-glycosidic carbohydrate fractions were desalted and analyzed by 500-MHz ^{1}H-

NMR spectroscopy. The same procedure was used to liberate, fractionate and identify the carbohydrate chains of free α- and β-subunits.

Liberation of the N-Linked Oligosaccharides by Hydrazinolysis

Hydrazinolysis was carried out according to [4]. Briefly, thoroughly dried hCG (10 mg) was suspended in 0.6 ml anhydrous hydrazine and heated for 8 h at 100°C. After evaporation of hydrazine, the material was *N*-reacetylated and reduced. For the reduction with ^{3}H-labeled $NaBH_4$, one sixth of the sample was dissolved in 0.2 ml 0.08 M NaOH and treated with $NaBH_4$ containing 1.5 mCi NaB^3H_4 in 0.185 ml *N,N*-dimethylformamide. The remaining part was reduced with NaB^2H_4. To facilitate the detection of oligosaccharide-alditols in further purification procedures, 1.0×10^5 cpm of the radioactive oligosaccharide mixture was added to the sample. Paper electrophoresis (Whatman 3MM paper, 4 kV, 70 V/cm) was carried out using a pyridine/acetic acid/water buffer, 3/1/387 by vol, pH 5.4. The oligosaccharide-alditols were recovered from the paper by elution with water.

Sugar Analysis

Sugar analysis was carried out by GLC on a capillary CPsil 5 WCOT fused silica column (25 m × 0.34 mm i.d., Chrompack, Middelburg, The Netherlands) using a Varian Aerograph 3700 gas chromatograph. The trimethylsilylated methyl glycosides were prepared by methanolysis, *N*-(re)acetylation and trimethylsilylation [23].

Methylation Analysis

Methylation analysis on 0.1 mg oligosaccharide-alditol was carried out essentially as described by Waeghe *et al.* [24]. The permethylated material was hydrolyzed in 4 M trifluoroacetic acid. Combined GLC-MS was performed on a Carlo Erba GC/Kratos MS 80/Kratos DS 55 system; electron energy, 70 eV; accelerating voltage, 2.7 kV; ionizing current, 0.1 mA; ion source temperature, 224°C; capillary BP 1 WCOT fused silica column (25 m × 0.33 mm i.d.; S.G.E., Ringwood, Australia).

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, Milwaukee, WI, USA) at p^{2}H 7 and room temperature. 500-MHz ^{1}H-NMR Spectra were recorded using a Bruker WM-500 spectrometer (SON hf-NMR facility, Department of Biophysical Chemistry, University of Nijmegen, The Netherlands) operating in the pulsed Fourier-transform mode at a probe temperature of 26°C[25]. Resolution enhancement of the spectra was achieved by Lorentzian-to-Gaussian transformation [26]. Selective suppression of the HO^{2}H-line was performed according to [27]. Chemical shifts are expressed in ppm downfield from internal sodium 4,4-dimethyl-4-silapentanesulfonate, 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.

Table 1. Molar carbohydrate composition of hCG, β-subunit and α-subunit

Monosaccharide	hCG	hCG-β	hCG-α
Fuc	0.3	0.9	0
Gal	2.4	5.4	2.0
Man[a]	3	3	3
GlcNAc[b]	4.7	5.4	3.4
GalNAc	0.9	2.7	0
NeuAc	3.3	7.2	1.1
Xyl[c]	0.2	0.3	0

[a] Mannose taken as 3.0

[b] Corrected for the amount of Asn-linked *N*-acetylglucosamine that is not cleaved under the conditions of methanolysis 23

[c] Xylose can be considered as a contaminant

EIA

Sandwich enzyme immunoassays were carried out as follows: 0.1 ml samples (approx. 50 mIE hCG, α- or β-subunit) were incubated on microtiter plates in the dark (2 h, room temperature) with immobilized monoclonal mouse anti-α- or anti-β-subunit antibodies. The complex formed was washed and conjugated with 0.1 ml polyclonal horseradish peroxidase-labeled mouse anti-hCG antibody (90 min, room temperature). The wells were washed and the complex was detected by reaction with tetramethylbenzidine in the presence of urea peroxide. After addition of 4 M sulphuric acid a yellow derivative was formed which was measured spectrophotometrically.

Results

Highly purified urinary hCG gave only two bands on SDS-polyacrylamide gel electrophoresis [28]. The upper band coincided with free β-subunit and the lower band with free α-subunit (results not shown). No other bands were visible. The sugar analysis data of native hCG, hCG-β and hCG-α are presented in Table 1. In accordance with previously described results ([6] and references cited therein), neither *N*-acetylgalactosamine, nor fucose were found in the α-subunit.

The three samples were subjected to the degradation and working-up procedure as schematically depicted in Fig. 1. The amidase activity of PNGase-F was checked using human serum transferrin, yielding *N*-acetyllactosamine type of di- and tri-antennary oligosaccharides (cf. [29]).

N-Glycosidic Carbohydrate Chains

After treatment of 10 mg denatured hCG with immobilized PNGase-F, chromatography of the digest over Mono Q gave rise to four main fractions, denoted NN, N1, N2 and N3, respectively (Fig. 2). Fraction NN represents non-glycoprotein-derived neutral material.

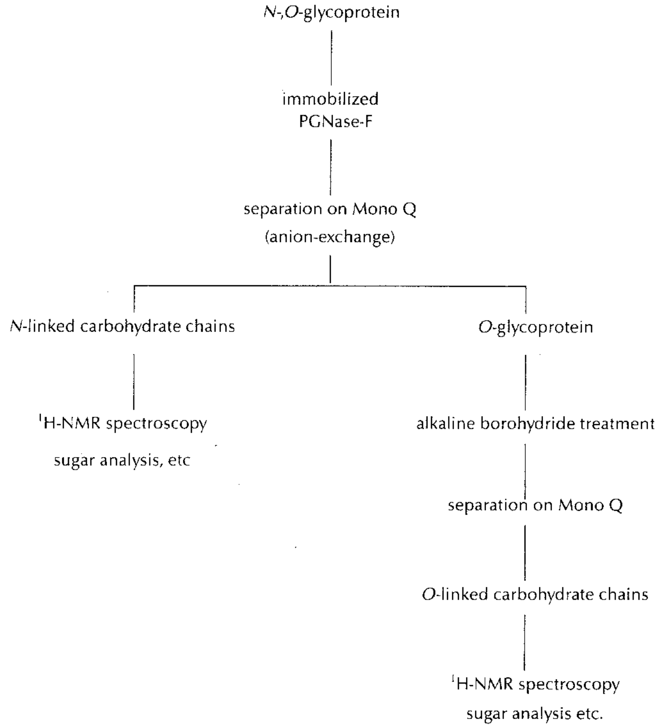
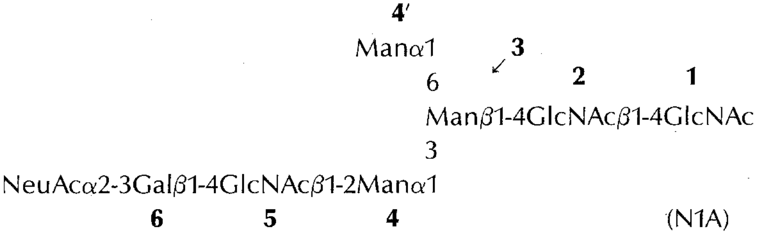


Figure 1. Schematic representation of the isolation procedure for *N*- and *O*-glycosidic carbohydrate chains of *N*-,*O*-glycoproteins.

N1 and N2 had the same elution volumes as reference di-antennary monosialo- and disialo- oligosaccharide-alditols obtained from human serum transferrin [29]. The retention time of fraction N3 was approximately the same as that of the protein-SDS complex from the model transferrin study (see above). The fractions N1 and N2 were analyzed by 500-MHz ^{1}H-NMR spectroscopy. The relevant NMR parameters, together with those of some reference compounds, are compiled in Table 2. Because the ^{1}H-NMR structural-reporter groups of N1 and N2 oligosaccharides, having a reducing chitobiose unit, have not been reported before, these data will be discussed in some detail.

The ^{1}H-NMR data of fraction N1 reveal that it contains mainly the following oligosaccharide (see [30]).



$$\begin{array}{llll}
 & \mathbf{4'} & & \\
 & \text{Man}\alpha1 \quad \swarrow\mathbf{3} & & \\
 & \quad 6 & \mathbf{2} & \mathbf{1} \\
 & \quad \text{Man}\beta1\text{-}4\text{GlcNAc}\beta1\text{-}4\text{GlcNAc} & & \\
 & \quad 3 & & \\
\text{NeuAc}\alpha2\text{-}3\text{Gal}\beta1\text{-}4\text{GlcNAc}\beta1\text{-}2\text{Man}\alpha1 & & & \\
\mathbf{6} \qquad\qquad \mathbf{5} \qquad\qquad \mathbf{4} & & &
\end{array}$$

(N1A)

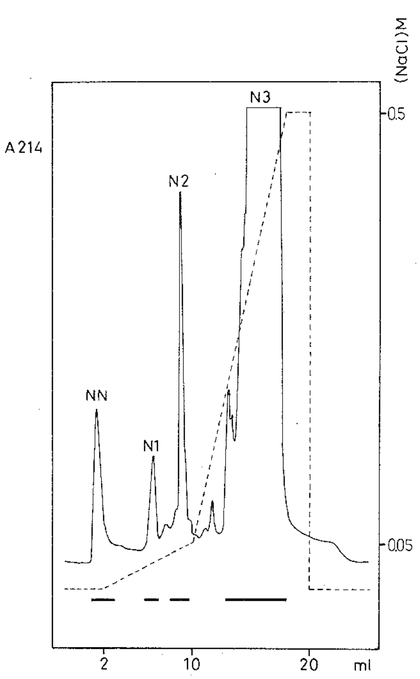
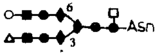
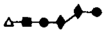
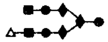
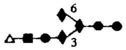


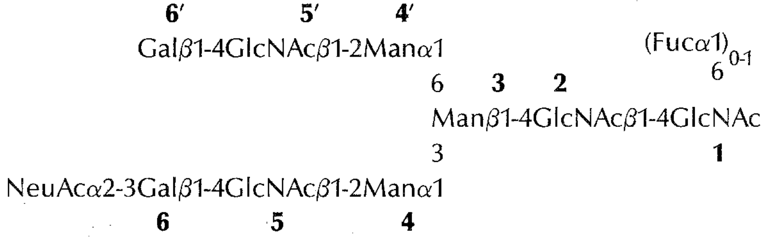
Figure 2. Fractionation pattern of the PNGase-F digestion products derived from hCG on an FPLC HR 5/5 Mono Q column.
PNGase-F digested hCG was desalted, lyophilized and dissolved in 0.7 ml water (HPLC quality). The column was eluted with a linear concentration gradient (----) from 0-50 mM NaCl in 8 ml water (HPLC quality), followed by a steeper gradient from 50-500 mM NaCl in 8 ml water at a flow rate of 60 ml/h. Injection volume 0.1 ml, detection 214 nm, sensitivity 1.0 AU. Fractions were collected as indicated.

The characteristic pattern of the Man-H-1 and H-2 signals prove the occurrence of the usual Manα1-6[Manα1-3]Manβ1-4 core [25]. The terminal position of Man-**4'** can be deduced from the typical chemical shift of its H-2 signal at δ=3.973 ppm (compare with reference structure **23** [25]). The latter assignment is based on selective ^{1}H-decoupling of Man-**4'** H-1. The presence of an α-(2-3) sialylated *N*-acetyllactosamine unit, linked β1-2 to Man-**4**, is evident from the combination of the position of the H-1 and H-2 signals of Man-**4**, the H-1 signal of GlcNAc-**5**, the H-1 and H-3 signals of Gal-**6**, and the H-3e and H-3a signals of NeuAc (compare with **36** [25], and [31]). In the area of the *N*-acetyl singlets the signals at δ = 2.031 ppm and 2.047 ppm belong to NeuAc and GlcNAc-**5**, respectively (compare with **35** and **36** [25]). Therefore, the additional signals at δ = 2.038 ppm and δ = 2.078 ppm represent the chitobiose GlcNAc-**1** and GlcNAc-**2** residues, respectively, in accordance with reference structure HST. The presence of GlcNAc-**1** in a reducing position (H-1α; δ = 5.187 ppm, $J_{1,2}$ = 3.5 Hz: H-1β; δ = 4.703 ppm, $J_{1,2}$ = 7.5 Hz) influences the chemical shift value of GlcNAc-**2** H-1. Because of the anomerization-effect, doublets for the GlcNAc-**2** H-1 are found at δ = 4.609 and 4.601 ppm. It is noteworthy, that there are no anomerization effects on the H-1 and H-2 signals of Man-**3**, nor on the NAc signals of GlcNAc-**1** and GlcNAc-**2** (see also [32]).

Low intensity signals at δ = 1.212 ppm, δ = 1.225 ppm, δ = 2.094 ppm and δ = 4.892 ppm indicate the presence of compound(s) in fraction N1 having Fucα(1-6) linked to GlcNAc-**1**. Small intensity signals at δ = 4.472 ppm and δ = 4.928 ppm suggest the

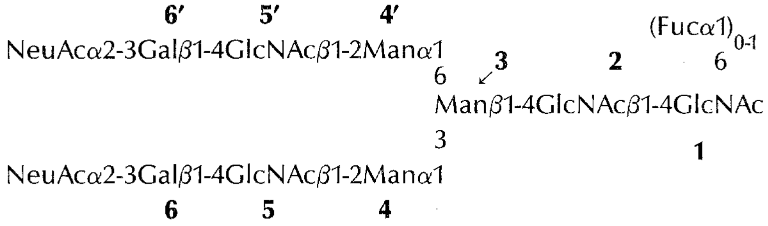
Reporter group	Residue[1]										
			45	38	N2A	N2B	HST	35	23	36	N1A
H-1	GlcNAc	1	5.068	-	α 5.188,β 4.694[2]	α 5.188, β 4.694	α 5.190 , β 4.696	-	-	-	α5.187, β4.703
	GlcNAc	2	4.682	α5.213, β~4.72	4.665[2]	α 4.611, β 4.601	α 4.615, β 4.606[2]	α 5.206, β~4.72	α5.215, β~4.72	α 5.214, β~4.72	α4.609, β4.601
	Man	3	~4.77	α4.775, β 4.763	4.763[3]	4.763[3]	n.d.	α~4.79, β~4.78	α4.792, β 4.784	α~4.77, β~4.76	4.761[4]
	Man	4	5.118	α5.122, β 5.120	5.116	5.116	5.134	5.122	5.140	α 5.123, β 5.119	5.119
	Man	4'	4.940	α4.923, β 4.928	4.923	4.923	4.947	-	4.921	α 4.929, β 4.932	4.916
	GlcNAc	5	4.575	4.578	4.572	4.572	4.606	4.579	4.606	4.579	4.570
	GlcNAc	5'	4.606	4.578	4.572	4.572	4.606	-	-	4.587	-
	Gal	6	4.545	4.544	4.543	4.543	4.443	4.544	4.446	4.546	4.542
	Gal	6'	4.447	α4.546, β 4.547	4.549	4.549	4.447	-	-	α 4.472, β 4.474	-
H-2	Man	3	4.253	α4.256, β 4.244	4.245	4.245	4.256	α4.245, β 4.234	α4.268, β 4.258	α 4.260, β 4.249	4.247
	Man	4	4.190	4.192	4.189	4.189	4.198	4.197	4.198	4.195	4.188
	Man	4'	~4.11	4.117	4.117[5]	4.117[5]	4.118	-	<4.0	4.117	3.973[5]
H-3	Gal	6	~4.11	4.113	4.112	4.112	n.d.	4.115	n.d.	4.115	4.112
	Gal	6'	<4.0	4.115	4.118	4.118	n.d.	-	-	<4.0	-
H-3a	NeuAc		1.800	1.797	1.797	1.797	1.718	1.799	1.719	1.796	1.796
	NeuAc'		1.717	1.799	1.801	1.801	1.718	-	-	-	-
H-3e	NeuAc		2.758	2.758	2.757	2.757	2.668	2.758	2.669	2.757	2.757
	NeuAc'		2.671	2.758	2.757	2.757	2.676	-	-	-	-
NAc	GlcNAc	1	2.012	-	2.039	2.039	2.037	-	-	-	2.038
	GlcNAc	2	2.094	α2.061, β 2.058	2.096	2.082	2.083	α2.043, β 2.041	α2.059, β 2.055	α2.060, β 2.057	2.078
	GlcNAc	5	2.048	2.049	2.048	2.048	2.069	2.049	2.070	2.049	2.047
	GlcNAc	5'	2.066	α2.046, β 2.043	2.043	2.043	2.066	-	-	α 2.049, β 2.047	-
	NeuAc		2.032	2.032	2.031	2.031	2.030	2.029	2.030	2.031	2.031
	NeuAc'		2.030	2.032	2.031	2.031	2.030	-	-	-	-
H-1	Fucα1-6		4.876	-	4.892	-	-	-	-	-	-
H-5	Fucα1-6		~4.12	-	4.100[6]	-	-	-	-	-	-
CH_3	Fucα1- 6		1.202	-	α1.212, β 1.223	-	-	-	-	-	-

presence of terminal Galβ(1-4) linked to GlcNAc at the Manα1-6 arm, as indicated in [30]. From the relative intensities of these signals it can be deduced that approximately 5% of fraction N1 has the following structure:



(N1B)

As is evident from comparison with the ^{1}H-NMR data of reference compounds **38**, **45** [25] and HST (see Table 2), fraction N2 contains



(N2A: with Fuc; N2B: without Fuc)

The structural-reporter groups of the di-antennary α(2-3)-sialylated outer chains and the mannotriose core unit are essentially the same as those reported for reference compound **38** [25]. The partial occurrence of an α(1-6)-linked fucose residue on GlcNAc-**1** of the reducing *N,N′*-diacetylchitobiose unit (approximately 30%) is reflected by the detection of the typical signals for H-1 (δ = 4.892 ppm; $J_{1,2}$ = 3.5 Hz), H-5 (δ = 4.100 ppm) and

---

Table 2. ^{1}H-Chemical shifts of structural-reporter-group protons of the constituent monosaccharides for the oligosaccharides N1A, N2A and N2B derived from hCG, together with those for the reference compounds **23, 35, 36, 38, 45** [25], and HST (derived from human serumtransferrin). Chemical shifts are given at 300 K, in ppm downfield from internal sodium 4,4-dimethyl-4-silapentanesulfonate in ^2H_2O. Compounds are represented by short-hand symbolic notation [25]; ●, GlcNAc; ◆, Man; □, Fuc; ■, Gal; △, NeuAcα2-3; ○, NeuAcα2-6.

[1] For numbering of the monosaccharide residues, see text.
[2] Tentative assignment.
[3] Ppm-value obtained *via* selective suppression of the HO^{2}H-line.
[4] Ppm-value obtained at 333K (NeuAc kept at δ = 2.031 ppm)
[5] Ppm-value obtained by selective irradiation of Man **4′** H-1.
[6] Ppm-value obtained by selective irradiation of Fuc CH_3.

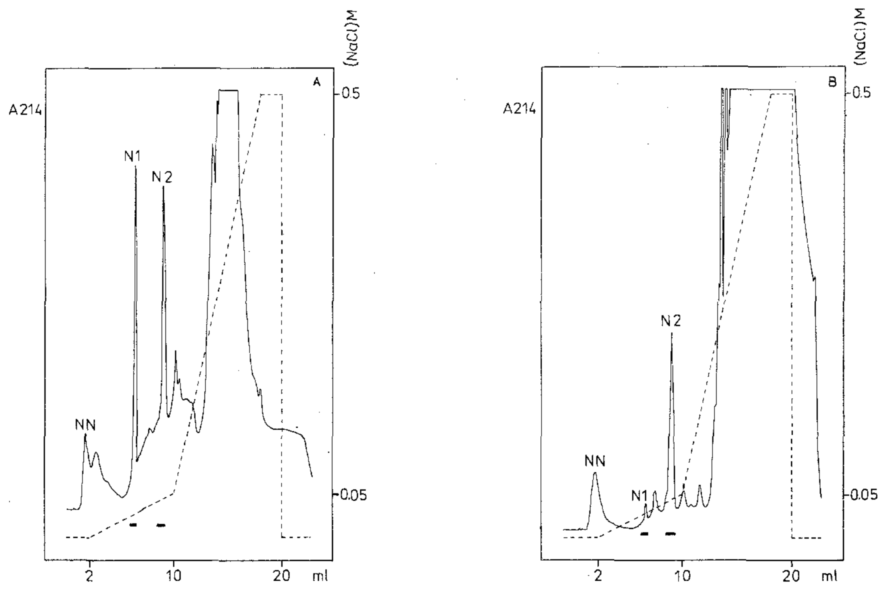


Figure 3. Fractionation pattern of the PNGase-F digestion products derived from α-(A) and β-subunit (B) of hCG on an FPLC HR 5/5 Mono Q column. Sensitivity 0.2 AU. For further details see Fig. 2.

CH_3 (δ = 1.223 ppm, β-anomer; δ = 1.212 ppm, α-anomer; $J_{5,6}$ = 6.5 Hz). The chemical shift of Fuc H-5 was determined by selective decoupling of Fuc CH_3. This series of structural-reporters forms a new set, being different from the sets described earlier for α(1-6)-linked fucose at GlcNAc-**1** in glycopeptide structures (H-1, δ = 4.876 ppm; H-5, δ = 4.125 ppm; CH_3, δ = 1.207 ppm [25]) and at GlcNAc-**1**-OL in oligosaccharide-alditol structures (H-1, δ = 4.896 ppm; H-5, δ = 4.071 ppm; CH_3, δ = 1.224 ppm [33]). The reducing *N,N'*-diacetylchitobiose is represented by the GlcNAc-**1** and GlcNAc-**2** H-1 and NAc signals. The GlcNAc H-1α and H-1β signals are present at δ = 5.188 ppm ($J_{1,2}$ = 3.3 Hz) and δ = 4.694 ppm ($J_{1,2}$ = 7.3 Hz), respectively. Because of the partial fucosylation two GlcNAc-**2** H-1 sets are observed. For the non-fucosylated structureN2B the two doublets (anomerization-effect) for GlcNAc-**2** H-1 are found at δ = 4.611 ppm ($J_{1,2}$ = 7.8 Hz) and δ = 4.601 ppm ($J_{1,2}$ = 7.8 Hz). The fucosylated structure N2A shows a doublet for GlcNAc-**2**- H-1 at δ = 4.665 ppm ($J_{1,2}$ = 7.8 Hz). The difference in chemical shifts between the two sets is comparable with that observed earlier for non-fucosylated and α(1-6)-fucosylated glycopeptides ($\Delta\delta$ = +0.068) [25]. There are no indications that fucosylation influences the resonance position of the GlcNAc-**1** NAc signal. The same phenomenon was found for carbohydrate chains terminated by GlcNAc-**1**-OL [29, 33] (see also [25] for glycopeptides). On the other hand the position of the GlcNAc-**2** NAc signal is influenced in a similar way as reported for glycopeptides and oligosaccharide-alditols [25, 29, 34]. The NAc signal of GlcNAc-**2** in the fucosylated oligosaccharide (δ = 2.096 ppm) is shifted 0.014 ppm upfield inthe non-fucosylated compound (δ = 2.082 ppm). Apart from α(2-3)-linked NeuAc, a few per cent of the di-antennary disialo compound with the NeuAc group in α2-6 linkage to Gal-**6**/**6'** also occurs, as is indicated by the chemical shifts at δ = 1.718 ppm (H-3a of NeuAc/NeuAc'), δ = 2.669 ppm (H-3e of NeuAc/NeuAc'), δ = 4.450

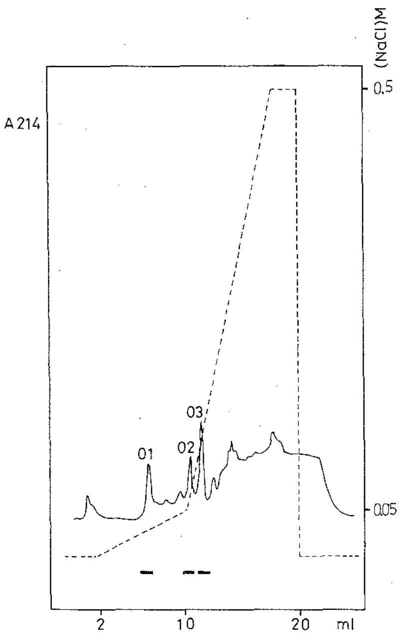
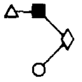
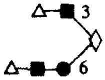


Figure 4. Fractionation pattern of the β-elimination products derived from *N*-deglycosylated hCG on an FPLC HR 5/5 Mono Q column. Sensitivity 0.2 AU. For further details see Fig. 2.

ppm (H-1 of Gal-**6**/Gal-**6'**), δ = 4.946 ppm (H-1 of Man-**4'**), δ = 5.137 ppm (H-1 of Man-**4**) and δ = 4.775 ppm (H-1 of Man-**3**) [25].

To ascertain the distribution of the two types of *N*-glycosidic chains over the α- and β-subunit, these subunits were prepared and the carbohydrate chains were released and characterized as described for intact hCG. The Mono Q elution profiles of the carbohydrate and protein material of the α- and β-subunits derived from 6 mg hCG are depicted in Fig. 3A and 3B, respectively. Fractions N1 and N2 have the same elution volumes as the corresponding fractions derived from native hCG (Fig. 2) and were proved to be compounds N1A and N2B (α-subunit) and N1A and N2A + N2B (β-subunit) by 500-MHz ^{1}H-NMR spectroscopy. Based on the UV-absorption in the Mono Q pattern the molar ratio of mono-antennary monosialo and di-antennary disialo compounds (compounds N1A and N2A/N2B, respectively) is approximately 1:1 in the α-subunit, while in the β-subunit the ratio is approximately 1:4. The occurrence of the mono-antennary monosialo compound N1A in the β-subunit cannot be ascribed to contamination of the β-subunit fraction with α-subunit or intact hCG, as PAGE and EIA showed the α- and β-fractions to be essentially pure. Based on the intensities of the relevant fucose ^{1}H-NMR signals, 50% of the di-antennary disialo compounds of the β-subunit are fucosylated. No fucose signals could be detected in the NMR-spectra of mono-antennary monosialo and di-antennary disialo compounds derived from the α-subunit which is in agreement with the sugar analysis data and earlier published work [6, 30, 35].

Table 3. ^{1}H-Chemical shifts of structural-reporter-group protons of the constituent monosaccharides for the oligosaccharide-alditols 01, 02 and 03 obtained from hCG, together with those for the reference compounds A [36], B [36] and C [7]. 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 NeuAc residues, by indicating the type of linkage of the neighbouring residue in the sequence. For further details, see Table 2;◆, GalNAc-OL.

Reporter-group	Residue						
		A	O1	B	O3	C	O2
H-2	GalNAc-ol	4.390	4.388	4.378	4.378	4.387	4.388
H-3		4.074	4.069	4.067	4.068	4.066	4.064
H-4		3.498	3.496	3.524	3.523	3.441	3.442
H-5		4.187	4.186	4.240	4.238	4.265	4.264
H-6		3.68	n.d.	3.84	n.d.	3.928	n.d.
H-6'		3.65	n.d.	3.475	3.471	n.d.	n.d.
NAc		2.046	2.044	2.042	2.042	2.065	2.065
H-1	Gal^3	4.547	4.548	4.541	4.542	4.529	4.532
H-3		4.122	4.120	4.117	4.115	4.113	4.115
H-4		3.931	3.929	3.927	3.927	3.928	n.d.
H-1	GlcNAc	-	-	-	-	4.550	4.551
H-6		-	-	-	-	4.004	4.007
NAc		-	-	-	-	2.062	2.062
H-1	Gal^4	-	-	-	-	4.545	4.546
H-3		-	-	-	-	4.113	4.115
H-4		-	-	-	-	3.956	3.956
H-3a	NeuAc^{3,3}	1.800	1.799	1.800	1.799	1.800	1.800
H-3e		2.774	2.771	2.774	2.775	2.775	2.777
NAc		2.034	2.033	2.032	2.032	2.033	2.032
H-3a	NeuAc^6	-	-	1.692	1.692	-	-
H-3e		-	-	2.723	2.725	-	-
NAc		-	-	2.032	2.032	-	-
H-3a	NeuAc^{3,4}	-	-	-	-	1.800	1.800
H-3e		-	-	-	-	2.755	2.753
NAc		-	-	-	-	2.031	2.032

O-Glycosidic Carbohydrate Chains

After the Asn-linked oligosaccharides had been removed from the glycoprotein, the *O*-glycosidic chains were liberated by alkaline borohydride treatment. The reaction products were separated on Mono Q and the three main fractions 01, 02, and 03 (Fig. 4) were analyzed by ^{1}H-NMR spectroscopy.

The ^{1}H-NMR data of the structural-reporter groups, together with those of reference compounds A, B [36] and C [7], are presented in Table 3. From these data it can be concluded that fraction 01 contains, as the only carbohydrate, the trisaccharide

NeuAcα2-3Galβ1-3GalNAc-OL

fraction 02 the hexasaccharide

NeuAcα2-3Galβ1
3
GalNAc-OL, (02)
6
NeuAcα2-3Galβ1-4GlcNAcβ1

and fraction 03 the tetrasaccharide

NeuAcα2-3Galβ1
3
GalNAc-OL, (03)
6
NeuAcα2

Based on the UV-absorption in the Mono Q pattern the molar ratio of 01, 02 and 03 amounts to 3:1:3.

Discussion

In this paper a general strategy for the separate isolation of oligosaccharide chains from *N*-,*O*-glycoproteins has been described. The reported combination of an enzymatic approach for the release of all types of *N*-linked chains (PNGase-F) and a chemical approach for the release of all types of *O*-linked chains, eliminates the problems which can arise when only chemical methods or not generally applicable enzymatic methods are used (see introduction). For the application of PNGase-F, the following items are of importance. An efficient procedure to check the activity of the enzyme is necessary. The liberation of the *N*-linked carbohydrate chains by the PNGase-treatment can be determined by a difference assay on the amino acid analyzer, by SDS-polyacrylamide gel-electrophoresis or by sugar analysis on the remaining protein fraction after PNGase-treatment. For instance, sugar analysis on part of fraction N3 (representing the protein-SDS complex resulting after PNGase-F digestion) revealed that, apart from the mucin type of carbohydrate chains, a few per cent of *N*-glycosidic material remained on the PNGase-F digested glycoprotein. Indeed upon a second incubation on the PNGase-F column some additional di-antennary disialo oligosaccharides (denoted structures N2A/N2B) were released, but no monosialo carbohydrate chains were detected.

In previous studies [30, 37] it was reported that the α-subunit contains a substantial amount of the fucose-free derivative of compound N1B, whereas the β-subunit was reported to contain both the fucose-containing and fucose-free derivatives of compound N1B. However, in our approach only traces of the di-antennary monosialo carbohydrate structures N1B could be detected. In a more recent publication of Mizuochi *et al.* [38] it remains unclear whether the di-antennary structures are fully sialylated or not. To exclude that the almost complete absence of Asn-linked sugar chains with galactose in a terminal position was the result of a contamination of the PNGase-F column with β-galactosidase and β-*N*-acetylhexosaminidase activities (which would degrade structure N1B to structure N1A), a control experiment was carried out. Incubation of *p*-nitrophenyl-β-D-galactopyranoside on the PNGase-F column under the same conditions as the glycoprotein did not yield free galactose. When *p*-nitrophenyl-β-D-galactopyranoside was incubated on a Sepharose-4B column with immobilized β-galactosidase, galactose was released completely. Liberation of the Asn-linked sugar chains *via* the hydrazinolysis procedure [4] and subsequent analysis of the isolated products by 500-MHz ^{1}H-NMR spectroscopy and methylation analysis revealed the presence of structure N1B in a higher yield. Recently, Nilsson *et al.* [39] reported that approximately half of the di-antennary carbohydrate chains of the α-subunit terminate in GlcNAcβ1-2Man and the other half in NeuAcα2-3Galβ1-4GlcNAc. These data are in contrast with our results and the results reported earlier [30].

Previously it has been reported that hCG contains a total of four identical carbohydrate chains linked to the Ser residues 121, 127, 132 and 138, having the structure of compound 03 [40]. Recently, Cole *et al.* [41] reported the occurrence of four different *O*-linked carbohydrate structures. The presence of the tri-, tetra- and hexasaccharide was confirmed by our studies, but we could not detect the minor disaccharide NeuAcα2-6GalNAc-OL that was stated to account for approximately 10% of the total *O*-glycosidic material. The anomeric configuration of the *N*-acetylglucosamine residue in the hexasaccharide was not determined in [41]. The NMR-data in this report demonstrate that the latter *N*-acetylglucosamine residue is in the β-linkage.

Di-antennary glycans have been proposed to occur in two rotameric forms in a ratio 1:1, in one of which the α1-6 branch is folded back on the core, positioning Gal-**6'** in close proximity to GlcNAc-**2** [25, 42]. Indications for backfolding in di-antennary oligosaccharides with GlcNAc-**2** in a terminal (reducing) position, are the anomerization effects apparent on the Gal-**6'** residue. In this paper it is shown that when the carbohydrate structure is extended with GlcNAc-**1**, anomerization effects on Gal-**6'** are no longer visible, confirming that backfolding of the α1-6 branch is not extended to the GlcNAc-**1** residue.

Acknowledgements

We thank Dr. J M Pekelharing for his advice concerning the immobilization of enzymes and Dr. J.A. van Kuik for recording the ^{1}H-NMR spectra. This work was supported by the Netherlands Foundation for Chemical Research (SON) with financial aid from the Netherlands Organization for the Advancement of Pure Research (ZWO).

References

1 Nilsson B (1978) Ph.D. Thesis, University of Lund, Sweden.
2 Ogata SI, Lloyd KO (1982) Anal Biochem 119:351-59.
3 Spiro RG (1976) Methods Carbohydr Chem 7:185-90.
4 Takasaki S, Mizuochi T, Kobata A (1982) Methods Enzymol 83:263-68.
5 Zinn AB, Plantner JJ, Carlson DM (1977) in The Glycoconjugates, Vol. I, eds. Horowitz MI, Pigman W, Academic Press, New York, p 69-85.
6 Berger EG, Buddecke E, Kamerling JP, Kobata A, Paulson JC, Vliegenthart JFG (1982) Experientia 38:1129-62.
7 Korrel SAM, Clemetson KJ, van Halbeek H, Kamerling JP, Sixma JJ, Vliegenthart JFG (1984) Eur J Biochem 140:571-76.
8 Egge H, Peter-Katalinic J, Paz-Parente J, Strecker G, Montreuil J, Fournet B (1983) FEBS Lett 156:357-62.
9 Michalski J-C, Peter-Katalinic J, Egge H, Paz-Parente J, Montreuil J, Strecker G (1984) Carbohydr Res 134:177-89.
10 Mutsaers JHGM, van Halbeek H, Kamerling JP, Vliegenthart JFG (1985) Eur J Biochem 147:569-74.
11 Bendiak B, Cumming DA (1985) Carbohydr Res 144:1-12.
12 Kobata A (1979) Anal Biochem 100:1-14.
13 Plummer TH Jr., Elder JH, Alexander S, Phelan AW, Tarentino AL (1984) J Biol Chem 259:10700-4.
14 Tarentino AL, Gómez CM, Plummer TH Jr. (1985) Biochemistry 24:4665-71.
15 Green ED, van Halbeek H, Boime I, Baenziger JU (1985) J Biol Chem 260:15623-30.
16 Chu FK (1986) J Biol Chem 261:172-77.
17 Steube K, Gross V, Hösel W, Tran-Thi T-A, Decker K, Heinrich PC (1986) Glycoconjugate J 3:247-54.
18 Lee K-O, Gesundheit N, Chen H-C, Weintraub BD (1986) Biochem Biophys Res Commun 138:230-37.
19 Swedlow JR, Matteri RL, Papkoff H (1986) Proc Soc Exp Biol Med 181:432-37.
20 Takahashi N, Nishibe H (1978) J Biochem (Tokyo) 84:1467-73.
21 Risley JM, van Etten RL (1985) J Biol Chem 260:15488-94.
22 Sugiyama K, Ishihara H, Tejima S, Takahashi N (1983) Biochem Biophys Res Commun 112:155-60.
23 Kamerling JP, Vliegenthart JFG (1982) Cell Biol Monogr 10:95-125.
24 Weaghe TJ, Darvill AG, McNeil M, Albersheim P (1983) Carbohydr Res 123:281-304.
25 Vliegenthart JFG, Dorland L, van Halbeek H (1983) Adv Carbohydr Chem Biochem 41:209-374.
26 Ernst RR (1966) Adv Magn Res 2:1-135.
27 Haasnoot CAG (1983) J Magn Reseon 52:153-58.
28 Laemmli UK (1970) Nature 227:680-85.
29 Van Pelt J, Damm JBL, Kamerling JP, Vliegenthart JFG (1987) Carbohydr Res, in press.
30 Endo Y, Yamashita K, Tachibana Y, Tojo S, Kobata A (1979) J Biochem (Tokyo) 85:669-79.
31 Gleeson PA, Feeney J, Hughes RC (1985) Biochemistry 24:493-503.
32 Baussant T, Strecker G, Wieruszeski J-M, Montreuil J, Michalski J-C (1986) Eur J Biochem 159:381-85.

33 Cahour A, Debeire P, Hartmann L, Montreuil J, van Halbeek H, Vliegenthart JFG (1984) FEBS Lett 170:343-49.
34 Mutsaers JHGM, Kamerling JP, Devos R, Guisez Y, Fiers W, Vliegenthart JFG (1986) Eur J Biochem 156:651-54.
35 Kessler MJ, Reddy MS, Shah RH, Bahl OP (1979) J Biol Chem 254:7901-8.
36 Van Halbeek H, Dorland L, Vliegenthart JFG, Fiat A-M, Jollés P (1981) FEBS Lett 133:45-50.
37 Mizuochi T, Kobata A (1980) Biochem Biophys Res Commun 97:772-78.
38 Muzuochi T, Nishimura R, Taniguchi T, Utsunomiya T, Mochizuki M, Derappe C, Kobata A (1985) Jpn J Cancer Res 76:752-59.
39 Nilsson B, Rosen SW, Weintraub BD, Zopf DA (1986) Endocrinology 119:2737-43.
40 Kessler MJ, Mise T, Ghai RD, Bahl OP (1979) J Biol Chem 254:7909-14.
41 Cole LA, Birken S, Perini F (1985) Biochem Biophys Res Commun 126:333-39.
42 Carver JP, Brisson J-R (1984) in Biology of Carbohydrates, Vol 2, eds. Ginsburg V, Robbins PW, Wiley, New York, p 289-331.