Carbohydrate Research, 236 (1992) 145–164
Elsevier Science Publishers B.V., Amsterdam

Heterogeneity of bovine lactotransferrin glycans. Characterization of α-D-Gal*p*-(1 → 3)-β-D-Gal- and α-NeuAc-(2 → 6)-β-D-Gal*p*NAc-(1 → 4)-β-D-GlcNAc-substituted N-linked glycans *

Bernadette Coddeville [a], Gérard Strecker [a], Jean-Michel Wieruszeski [a], Johannes F.G. Vliegenthart [b], Herman van Halbeek [b], Jasna Peter-Katalinić [c], Heinz Egge [c] and Geneviève Spik [a]

[a] *Laboratoire de Chimie Biologique, Unité Mixte de Recherche du Centre National de la Recherche Scientifique No. 111, Université des Sciences et Techniques de Lille Flandres – Artois, F-59655 Villeneuve d'Ascq (France)*

[b] *Department of Bio-Organic Chemistry, Bijvoet Center, Utrecht University, P.O. Box 80.075, NL-3508 TB Utrecht (Netherlands)*

[c] *Physiologisch-Chemisches Institut der Universität Bonn, Medizinische Fakultät, Nussallee 11, D-5300 Bonn 1 (Germany)*

(Received October 22nd, 1991; accepted December 6th, 1991)

ABSTRACT

Lactotransferrin isolated from a pool of mature bovine milk has been shown to contain *N*-glycosidically-linked glycans possessing *N*-acetylneuraminic acid, galactose, mannose, fucose, *N*-acetylglucosamine, and *N*-acetylgalactosamine. The glycopeptides obtained by Pronase digestion were fractionated by concanavalin A–Sepharose affinity chromatography into three fractions: slightly retained (A), retained (B), and strongly retained (C). The structure of the glycans of the three fractions has been determined by application of methanolysis, methylation analysis, fast atom bombardment-mass spectrometry, and ^{1}H NMR spectroscopy. Diantennary structures without GalNAc were present as partially sialylated and partially (1 → 6)-α-L-fucosylated structures in Fractions A and B. Sequences containing α-D-Gal*p*-(1 → 3)-β-D-Gal on the α-D-Man-(1 → 6) antenna, and β-D-Gal*p*NAc-(1 → 4)-β-D-GlcNAc and α-NeuAc-(2 → 6)-β-D-Gal*p*NAc-(1 → 4)-β-D-GlcNAc on the α-D-Man-(1 → 3) antenna were characterized in the oligosaccharide-alditols obtained by reductive cleavage of Fraction B. A series of Man_{4-9}-GlcNAc structures were identified in Fraction C after endo-*N*-acetyl-β-D-glucosaminidase digestion. These results show that the structures of bovine lactotransferrin glycans are more heterogeneous than those of previously characterized transferrin glycans.

INTRODUCTION

Mammalian milks are known to contain a variable amount of an iron-binding glycoprotein called lactotransferrin[1] or lactoferrin[2,3]. The first isolation of lacto-

Correspondence to: Professor G. Spik, Laboratoire de Chimie Biologique, Unité Mixte de Recherche du Centre National de la Recherche Scientifique No. 111, Université des Sciences et Techniques de Lille Flandres–Artois, F-59655 Villeneuve d'Ascq, France.

* Dedicated to Professor Jean Montreuil.

transferrin from bovine milk was described[4] in 1960. The sequence of its single polypeptide chain of 689 amino acids has been recently identified by cDNA analysis[5,6]. The similarity between this sequence and those of human[7,8] and murine[9] lactotransferrins suggests that bovine lactotransferrin, like the other members of the transferrin family[10,11], is a bilobal protein[12] containing one iron-binding site in each lobe. The glycoprotein nature of bovine milk lactotransferrin has been known[4,13] since 1960. Partial characterization of the structure of the glycans of bovine lactotransferrin has been previously reported[14–16]. We describe herein the complete structures of the glycans of the *N*-acetyllactosamine-type and oligomannose-type present in bovine lactotransferrin, as determined by methanolysis, methylation analysis, fast atom bombardment mass spectrometry, and ^{1}H NMR spectroscopy.

EXPERIMENTAL

Materials.—Standard oligosaccharides and enzyme. A mixture of oligomannosides, $Man_2GlcNAc$–$Man_9GlcNAc$, isolated by Strecker and Montreuil from the urine of patients with mannosidosis[19], was kindly provided by Dr. G. Strecker from our laboratory. Endo-*N*-acetyl-β-D-glucosaminidase (EC 3.2.1.96), isolated from the Basidiomyces *Sporotrichum dimorphosporum*, by Bouquelet et al.[18], and immobilized on Sepharose 4B according to Kol et al.[19], was kindly donated by Dr. S. Bouquelet from our laboratory.

^{1}H NMR spectroscopy.—For ^{1}H NMR spectroscopic analysis, glycopeptides and oligosaccharide-alditols were repeatedly treated with D_2O (99.95 atom%, from Commissariat à l'Énergie Atomique, Saclay, France) at room temperature and at pD 7 with intermediate lyophilization[20]. The deuterium-exchanged glycopeptides and oligosaccharide-alditols were submitted to ^{1}H NMR spectroscopy performed at 400 MHz on a Bruker AM 400-WB spectrometer (Centre Commun de Mesures, Université de Lille Flandres–Artois, Villeneuve d'Ascq, France) or at 500 MHz on a Bruker WM-500 (SON hf-NMR facility, Department of Biophysical Chemistry, University of Nijmegen, The Netherlands), equipped respectively with a Bruker Aspect-3000 computer or a Bruker Aspect-2000 computer at a probe temperature of 27°. Resolution enhancement was achieved by Lorentzian-to-Gaussian transformation from quadrature-phase detection, followed by employment of a 32K point complex FT. Chemical shifts (δ) are expressed downfield from the signal for internal sodium 4,4-dimethyl-4-silapentane-1-sulfonate, but were actually measured by reference to internal acetone (δ 2.225) with an accuracy of 0.002 ppm.

Mass spectrometry.—Fast atom bombardment-mass spectrometry (FABMS) was performed in the positive-ion mode on a ZAB-HF mass spectrometer (VG analytical, Manchester, UK) under conditions described by Egge and Peter-Katalinic[21]. Briefly, the permethylated glycan (5 μg) dissolved in MeOH (1 μl) was applied with a microsyringe to the stainless steel target precoated with 3-mercapto-1,2-propanediol (3 μL). The target was bombarded with Xe atoms of a kinetic energy

equivalent to 9 keV. The acceleration voltage was set to 7 keV in order to increase the mass range of the instrument above m/z 3500. In separate measurements, the target was precoated with 1% solution of NaOAc in MeOH (1 μL) in order to enhance $M + Na^+$ ion formation. Spectra were recorded in a mass-controlled scan and evaluated by counting the spectral lines.

Carbohydrate analysis.—Percentage compositions in total neutral sugars, hexosamines, and sialic acids were determined by colorimetric microprocedures[22]. Monosaccharide molar ratios were determined after methanolysis[23] and trimethylsilylation[24] modified as follows. A solution of glycopeptides, oligosaccharides, or oligosaccharide-alditols (corresponding to 2 μg of total sugars) in 0.5 M HCl in MeOH (500 μL) was heated in a screw-cap tube for 24 h at 80°. After cooling, the acid was neutralized by addition of $AgCO_3$ until pH 6–7 and the mixture was *N*-reacetylated by addition of acetic anhydride (10 μL). After one night at room temperature, the supernatant was dried under N_2, the monosaccharides were trimethylsilylated with bis(trimethylsilyl)trifluoroacetamide (20 μL) in the presence of pyridine (20 μL) for 2 h at room temperature. The methyl trimethylsilylglycosides were characterized and estimated by GLC. This was performed with a Girdel model 300 gas chromatograph equipped with a flame-ionization detector, and a glass capillary column (0.2 mm × 25 m) filled with 5% (w/w) Silicone OV 101 on Chromosorb W-HP (mesh 120–140); the carrier gas was H_2 at a flow rate of 10 mL/min and a pressure of 0.05 MPa, and the temperature was programmed from 120–240° at 2°/min.

Methylation analysis.—Glycopeptides and oligosaccharide-alditols were methylated by the Hakomori method[25], as modified by Paz-Parente et al.[26]. Briefly, glycopeptides and oligosaccharide-alditols (100 μg) were dissolved in dimethyl sulfoxide (100 μL) in Teflon-lined screw-cap tubes. Lithium methylsulfinyl carbanion (200 μL) was added under an inert atmosphere and the mixture was sonicated for 60 min. After cooling, methyl iodide (200 μL) was added and sonication repeated for 45 min. The methylation was terminated by addition of water and the permethylated products were extracted with $CHCl_3$. The organic phase was washed with water, dried, and concentrated. The permethylated compounds were methanolized and acetylated according to Fournet et al.[27]. The resulting mixture of methyl *O*-acetyl-*O*-methyl-glycosides was separated by GLC in a capillary column (0.3 mm × 25 m) coated with OV-101. The temperature was programmed from 120–240° at 3°/min. The monosaccharide derivatives were also identified by GLC–MS in a Ribermag R model 10-10 mass spectrometer (Riber, Rueil–Malmaison, France) coupled to a Sydar 121 data system.

Preparation of bovine milk lactotransferrin glycopeptides.—Lactotransferrin was isolated from a pool of mature bovine milk, collected from a local farm, under the conditions described by Chéron et al.[28]. The bovine lactotransferrin preparation, homogeneous in sodium dodecyl sulfate–poly(acrylamide) gel electrophoresis (SDS-PAGE), was reduced and alkylated[29]. The reduced and alkylated sample was exhaustively digested with Pronase[30] (Merck). The glycopeptides were purified by

gel filtration on a Bio-Gel P-2 column (2 × 60 cm). Elution was carried out with water and the fraction containing glycopeptides was monitored by an orcinol–H_2SO_4 reagent. The mixture of glycopeptides was fractionated on a ConA-Sepharose column (2 × 24 cm)[31], eluted successively with the equilibrating buffer: 5 mM sodium acetate, mM $MnCl_2$, mM $CaCl_2$, mM $MgCl_2$, 0.1 M NaCl, pH 6.5, and with an increasing linear gradient of methyl α-D-glucopyrannoside from 0 to 200 mM. Three fractions were obtained (A, B, and C). Methyl α-D-glucopyrannoside and salts were eliminated by gel-filtration chromatography on a Bio-Gel P-2 column (1.2 × 50 cm), and the glycopeptides were eluted with water and the solution was lyophilized.

Alkaline borohydride treatment.—Fraction B was reduced under the alkaline conditions used to release O-[32] and N-linked glycans[33]. In the latter procedure, glycopeptide Fraction B (3 mg) was dissolved in a solution containing M NaOH–M $NaBH_4$ (0.5 mL) and heated for 6 h at 100°. The reaction was stopped by addition of concd acetic acid until pH 4.5. The solution was applied on a Bio-Gel P-2 column (1.2 × 70 cm) and the oligosaccharide-alditols were eluted with distilled water and lyophilized. Subsequently, the oligosaccharide-alditols, redissolved in concd $NaHCO_3$ (100 μL), were *N*-reacetylated[34] by adding acetic anhydride (20 μL every 5 min, 5 times) and purified by gel filtration on a Bio-Gel P-2 column.

Endo-N-acetyl-β-D-glucosaminidase digestion.—Oligosaccharides were released from glycopeptide Fraction C (20 mg) by immobilized endo-*N*-acetyl-β-D-glucosaminidase[19]. The glycopeptides were passed through the column (1.61 munit of endo-*N*-acetyl-β-D-glucosaminidase activity; 25 mL of Sepharose-4B) equilibrated with 0.02 M phosphate–0.01 M citrate buffer, pH 5.0, for 48 h at 37°. The extent of hydrolysis was estimated by TLC on a silica gel plate (Kieselgel 60, Merck) in 2:1:2 (v/v) butanol–acetic acid–water; the carbohydrates as well as the standard oligosaccharides were stained with an orcinol–H_2SO_4 reagent. The sample was applied to a Bio-Gel P-2 column (1.5 × 60 cm) equilibrated with distilled water.

Isolation of oligosaccharides by LC.—The oligosaccharides obtained from glycopeptide Fraction C were analyzed with a Spectra-Physics Model 8750 liquid chromatograph, equipped with a Rheodyne 7105 injection valve and a Spectra-Physics Model 8450 detector operating at 206 nm. The chromatograms were recorded with a Spectra-Physics Model 4270 computer integrator. The chromatography was performed on an Amino AS-5A column (4 mm × 30 cm, Brownlee Labs, Inc.) equilibrated with the initial solvent (13:7, v/v, acetonitrile–water). The oligosaccharides were eluted[35] first with the equilibrating solvent for 40 min and then with a linear gradient of 1:1 (v/v) acetonitrile–water for 50 min. The flow rate was maintained at 1 mL/min.

RESULTS

Preparation and fractionation of the glycopeptides.—The purified sample of bovine lactotransferrin was characterized by SDS-PAGE as a single protein band

TABLE I

Molar carbohydrate compositions of bovine lactotransferrin and Con A-fractionated glycopeptides

Compound	Fuc	Gal	Man	GalNAc	GlcNAc	NeuAc
Bovine lactotransferrin [a]	0.2	0.4	4.3	0.4	2.0	0.2
Pronase glycopeptides [a]	0.2	0.5	4.6	0.4	2.0	0.3
Con A-Fraction A [b]	0.5	1.5	3.0		3.1	1.1
Con A-Fraction B [b]	0.7	1.6	3.0	0.8	3.3	0.9
Con A-Fraction C [a]			7.2		2.0	

[a] Molar ratios were calculated on the basis of two 2-acetamido-2-deoxyglucose units. [b] Molar ratios were calculated on the basis of three mannose units.

having a molecular mass of about 80000 daltons. The carbohydrate component, representing 11.2% of the purified lactotransferrin sample, consisted of 5.5% neutral sugars, 5.1% hexosamines, and 0.6% sialic acids. The sugars were identified as fucose, galactose, mannose, *N*-acetylgalactosamine, *N*-acetylglucosamine, and neuraminic acid by GLC of their trimethylsilyl derivatives (Table I). The molar ratio of these monosaccharides (Table I) was similar to that estimated in the mixture of glycopeptides obtained after Pronase digestion. Both carbohydrate compositions were characterized by a high content in mannose and *N*-acetylglucosamine residues and a lower content in fucose, galactose, *N*-acetylgalactosamine, and *N*-acetylneuraminic acid, suggesting the presence of different types of glycan structure. The results obtained by affinity-chromatograpy, on a Con A–Sepharose column, of the mixture of glycopeptides confirmed this hypothesis since 3 fractions, Con A-Fraction A, Con A-Fraction B, and Con A-Fraction C, were obtained in the ratio 11:8:81. The carbohydrate molar ratios of these three fractions (see Table I) suggested that the structures of the glycans of Con A-Fraction C are of the oligomannoside type, and those of the glycans present in Con A-Fraction A and Con A-Fraction B are of the *N*-acetyllactosamine type. Only the glycans of Fraction B were characterized by the presence of *N*-acetylgalactosamine residues.

Structure determination of Con A-Fraction A glycopeptides.—The primary structure of the glycans present in the glycopeptide Con A-Fraction A was established by methylation analysis in conjunction with 400 MHz ^{1}H NMR spectroscopy. The relative proportions of the methylated derivatives of the monosaccharides, taken from the mass-fragmentometry scans and combined with the molar carbohydrate composition, are given in Table II. Identification of 1.6 residues of 3,4,6-tri-*O*-methylmannoside in addition to 1 residue of 2,4-di-*O*-methylmannoside is interpreted in terms of the presence of a diantennary glycan. The occurrence of 2,3,4,6-tetra-*O*-methylgalactoside and 2,3,4-tri-*O*-methylgalactoside indicated that some galactose units are in an external position and others are substituted at O-6. The presence of permethylated neuraminic acid suggested that the galactose residue may be substituted by neuraminic acid.

In order to establish the complete primary structure of the glycans, the glycopeptides were submitted to 400-MHz ^{1}H NMR spectroscopy which indicated the

TABLE II

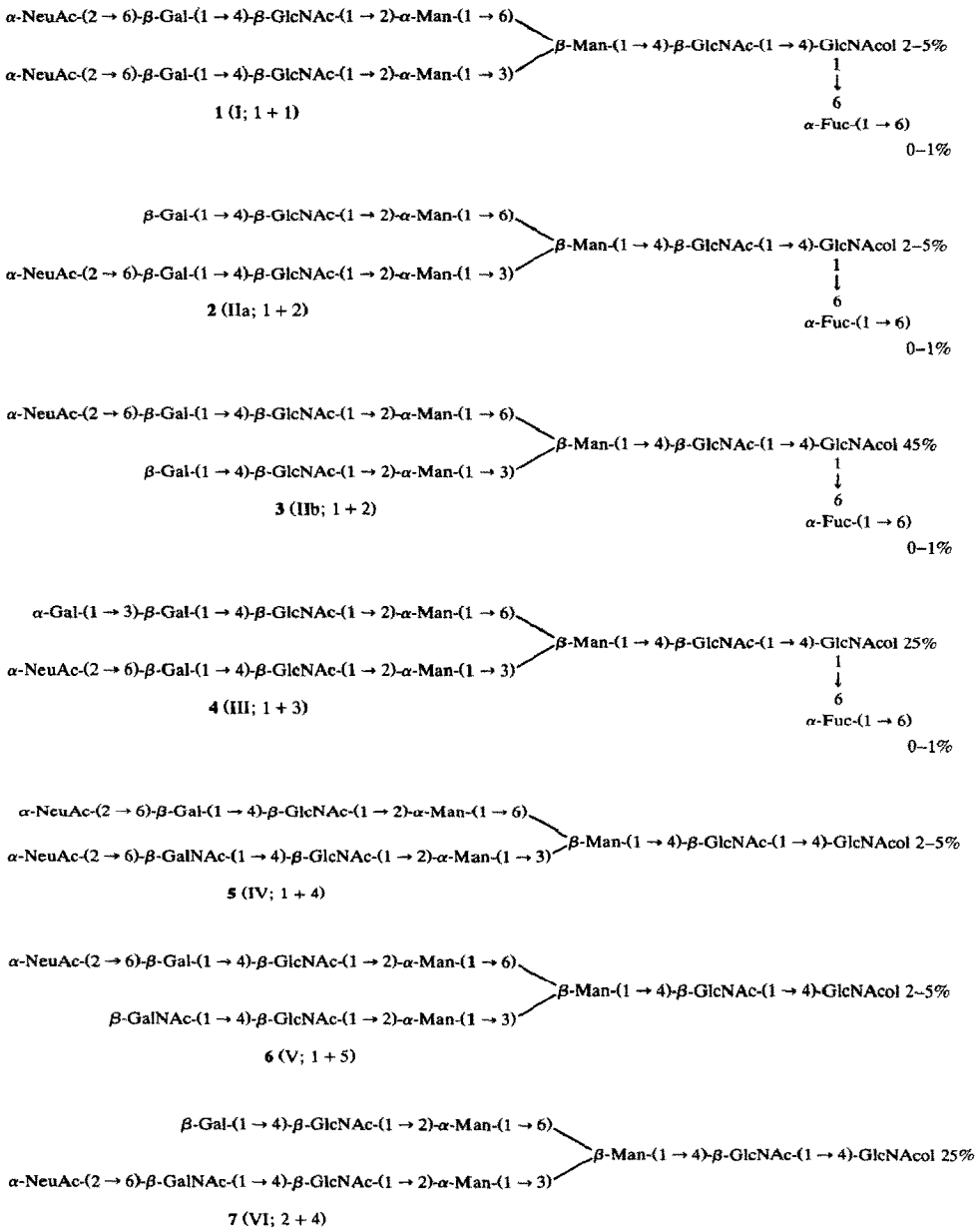
Mole ratios of methyl ethers present in permethylated Con A-glycopeptides Fraction A and in permethylated oligosaccharide-alditols isolated from Con A-Fractions B and C

Monosaccharide methyl ethers	Con A-Fraction A [a]	Con A-Fraction B [a]	Con A-Fraction C			
			C-I [b]	C-II [c]	C-III [c]	C-IV [c]
2,3,4-Me_3-Feuc	0.5	0.4				
2,3,4,6-Me_4-Gal	0.6	0.9				
2,4,6-Me_3-Gal		0.4				
2,3,4-Me_3-Gal	0.9	0.8				
3,4,6-Me_3-Man	1.6	2.0	1.7		0.8	1.9
2,4,6-Me_2-Man			1.0			
2,4-Me_2-Man	1.0	1.0	0.2	2.0	2.0	2.0
2,3,4,6-Me_4-Man			1.2	2.5	2.5	2.7
3,4,6-Me_3-GalNAcMe		0.2				
3,4-Me_2-GalNAcMe		0.8				
3,6-Me_2-GlcNAcMe	3.1	3.1				
1,3,5,6-Me_2-GlcNAcol			1.3	0.9	0.8	1.0
1,3,5-Me_3-GlcNAcol		[d]				
4,7,8,9-Me_4-NeuAcMe	1.1	0.8				

[a] Mole ratios were calculated on the basis of 1 residue of 2,4-Me_2-Man per mol of glycan. [b] Mole ratios were calculated on the basis of 1 residue of 2,4,6-Me_3-Man per mol of glycan. [c] Mole ratios were calculated on the basis of 2 residues 2,4-Me_2-Man per mol of glycan. [d] Traces.

presence of a diantennary *N*-acetyllactosamine structure partially fucosylated and sialylated. Indeed, the NMR parameters (not shown) agree with those described for the monofucosyl disialyl diantennary glycopeptides obtained from numerous glycoproteins[20,36]. In particular, the H-3*a* and H-3*e* signals of the NeuAc unit were observed to resonate at δ 1.717 and 2.668, which are characteristic of α-(2 → 6)-linked NeuAc groups. The H-1 protons of Man-*4* and Man-*4'*, respectively, showed two doublets. For Man-*4*, the H-1 signals at δ 5.136 and 5.120 are related to the sialo and asialo (1 → 3)-linked antennae present in the ratio 1:1, and a similar observation could be made for the Man-*4'* H-1 signal (sialo, δ 4.947; asialo, δ 4.928). The presence of an α-L-fucosyl group (1 → 6)-linked to GlcNAc^1 was verified by the occurrence of signals at δ 1.226 (Fuc CH_3) and 4.896 (Fuc H-1). The doubling of the NAc signals of GlcNAc (δ 2.080 and 2.088) in the ratio 3:2 indicated the presence of 0.4 group of Fuc. These results confirmed that the Con A-Fraction A contains a mixture of disialyl, monosialyl, and monofucosyl, diantennary *N*-acetyllactosamine glycopeptides, having a (2 → 6)-linked α-NeuAc and (1 → 6)-linked α-L-Fuc group.

Structure determination of Con A-Fraction B oligosaccharide-alditols.—As the presence of GalNAc residues in Con A-Fraction B glycopeptides suggested the existence of O-linked glycans, a reductive elimination procedure was applied to these glycans. However, under the conditions used, no traces of 2-acetamido-2-deoxygalactitol was identified. Therefore, the oligosaccharide-alditols were released from Con A-Fraction B by the reductive alkaline cleavage used to liberate N-linked glycans. The structures of these oligosaccharide-alditols **1–7** (Scheme 1)



Scheme 1. Structures of the oligosaccharide-alditols present in the Con A-Fraction B isolated from bovine lactotransferrin glycopeptides. The type of structure and the type of antenna are given in parentheses.

was established on the basis of the results obtained by methylation analysis (Table II), FABMS analysis (Tables III and IV; Fig. 1) and 400 MHz ^{1}H NMR spectroscopy (Table V, Fig. 2, and Scheme 2).

The molar ratios of the methyl derivatives of Gal, Man, Fuc, GlcNAc, and NeuAc were consistent with the presence of a diantennary structure of the *N*-acetyllactosamine-type partially sialylated and fucosylated. Occurrence of additional traces of 3,4,6-tri-*O*-methyl GalNAc and of 0.8 residue of 3,4-di-*O*-methyl GalNAc suggested the presence, in these diantennary glycans, of GalNAc residues located in the external position or substituted at O-6.

In order to define the complete structure of the Con A-Fraction B glycans, the oligosaccharide-alditols were analyzed by FABMS and the results obtained were compared to those given by ^{1}H NMR spectroscopy. As discussed in detail previously[21], permethylated *N*-acetyllactosamine type glycans furnish FABMS spectra (Fig. 1) that show intense fragment-ions representing the antenna, designed $A^+1 \rightarrow 6$, and fragment ions produced by cleavage of the *N*,*N*′-diacetylchitobiose component, one representing the reduced terminal residue, and the other, C^+ type, comprising one GlcNAc, the mannotriose core, and the antenna present. In Table III, the six major types of antennae are listed. As can be deduced from the C^+-type ions and the molecular ions ($M + Na^+$) listed in Table IV, the oligosaccharide-alditols present in Con A-Fraction B were only of the diantennary type. Some of the oligosaccharide-alditols possess however peculiar antennae, such as Hex → Hex → $HexNAc^+$ (*m/z* 668), HexNAc → $HexNAc^+$ (*m/z* 505), and NeuAc-HexNAc-$HexNAc^+$ (*m/z* 866) suggesting the occurrence of Gal → Gal → GlcNAc*, GalNAc → GlcNAc, and NeuAc → GalNAc → GlcNAc sequences. The major molecular ions ($M + Na^+$) at *m/z* 2446 and 2620 are characteristic of classical afuco- and fucosyl-monosialyl diantennary glycans. This finding is in agreement with the ^{1}H NMR analysis (Table V) which confirmed the presence of two major signals at δ 4.928 (H-1 of Man-*4′*; type *2*, asialo antenna) and δ 5.136 (H-1 of Man-*4*; type *1*, sialo antenna). The corresponding protons for Gal-*6* and Gal-*6′* were observed at δ 4.445 (sialo) and 4.471 (asialo). The intensities of the signals observed at δ 4.896 [H-1 of α-L-Fuc-(1 → 6)] and δ 1.221–1.226 (CH_3-Fuc) indicated the proportion of fucosyl groups on the GlcNAcol residue to be ~ 40%. The heterogeneity of the CH_3 signal, not observed in the spectrum of the native glycopeptide, is probably due to a chemical modification of the GlcNAcol residue occurring during the alkaline hydrolysis. The second type of structure with a sialyl group on the (1 → 6) antenna also exists, as could be deduced from the occurrence of signals at δ 5.123 (H-1 of asialo Man-*4*) and δ 4.946 (H-1 of asialo Man-*4′*, not indicated in Table V). Nevertheless, these parameters may also result from glycans of the (1 + 4) and (2 + 4) antennary-type (see structures **1–7** for type of antennae). Traces of glycans of the (1 + 1) antennary type could also be observed based on the observation of the molecular ions $M + Na^+$ at *m/z* 2807 (afuco) and 2981 (fuco). Therefore, the major compounds of the (1 + 2) antennary type corresponded to oligosaccharide-alditols **3** and **4**. The presence of glycans of the (1 + 3) antennary

* All sugars discussed have the D-configuration (except for L-fucose) and the pyranose form.

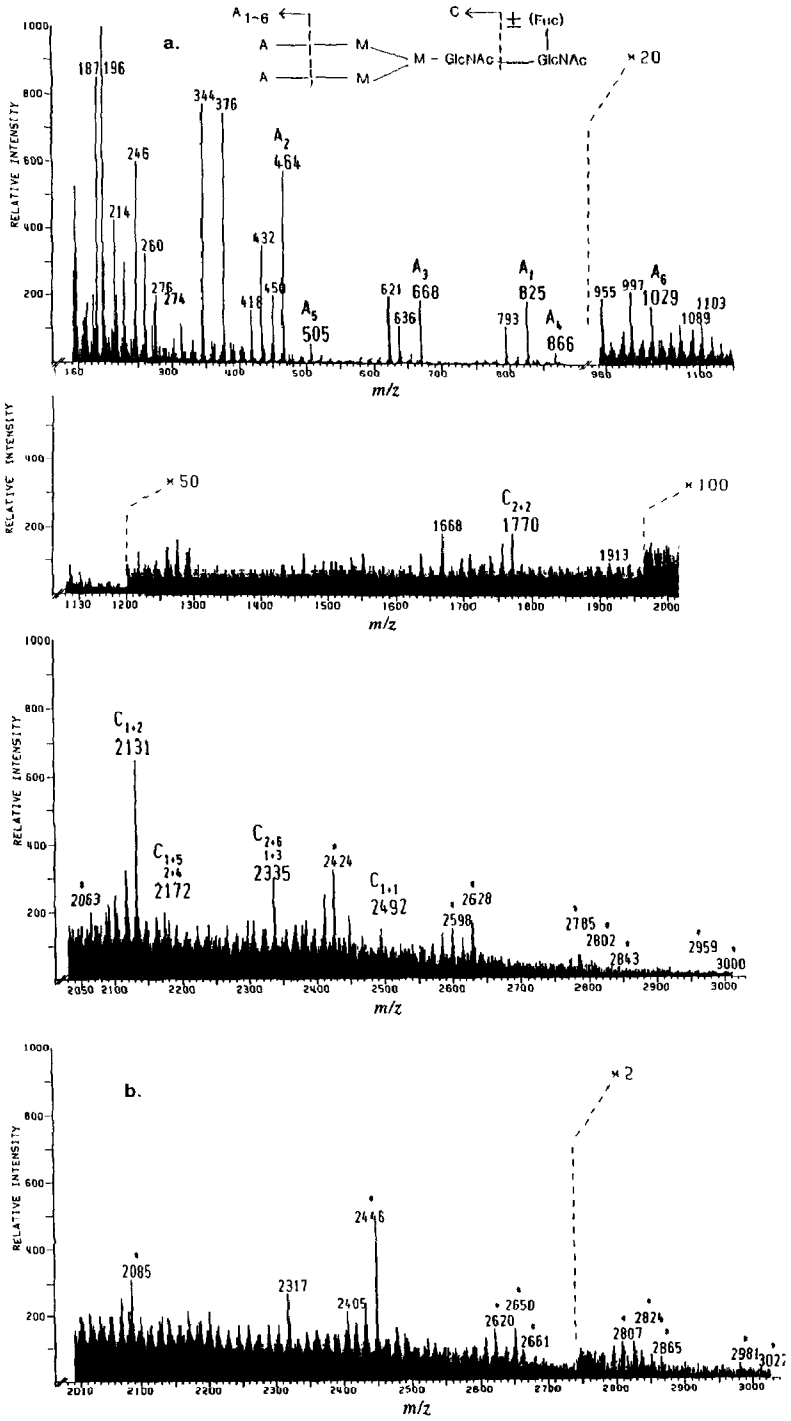


Fig. 1. (a) Positive-ion FABMS of the permethylated glycan Con A-Fraction B desorbed from a thioglycerol matrix. (b) The molecular-ion region of the spectrum recorded after addition of sodium acetate to the target. Fragment ions representing different antennae are designated A_{1-6}. Fragment ions produced by cleavage of the chitobiose unit comprising antennae and the mannotriose core are marked with C. Molecular ions $(M+H^+)$ or $(M+Na^+)$ are indicated by an asterisk.

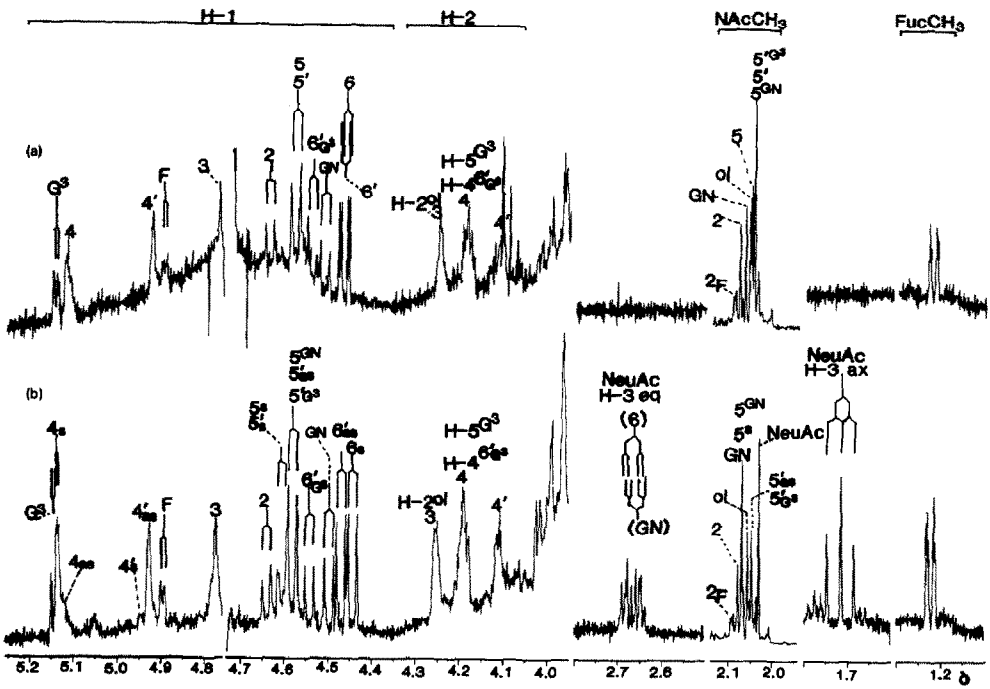
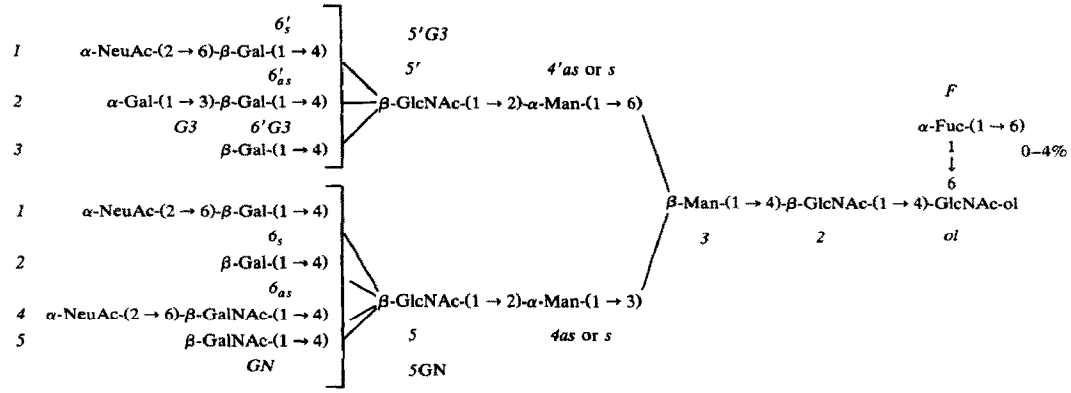


Fig. 2. Structural reporter group regions of the 400-MHz ^{1}H NMR spectrum of oligosaccharide-alditols isolated from the glycopeptides Con A-Fraction B of bovine lactotransferrin: (a) Fraction B sialylated. (b) Fraction B desialylated. Abbreviations: 4as, α-Ma-(1 → 3) residue of a desialylated antenna; 4's or 4'as, α-Man-(1 → 6) residue of a sialylated or desialylated antenna; 6_s, β-Gal substituted by α-NeuAc-(2 → 6); 6_{as}, terminal Gal; 6'G3, β-Gal substituted by β-Gal-(1 → 3); G3, terminal α-Gal-(1 → 3); 5GN, GlcNAc substituted by β-GalNAc-(1 → 4); 5'G3, GlcNAc substituted by the branch α-Gal-(1 → 3)-β-Gal-(1 → 4); GN, β-GalNAc-(1 → 4) residue; F, α-Fuc-(1 → 6) residue; H-5^{G3}, H-5 of α-Gal-(1 → 3); and H-4$^{6'G3}$, H-4 of α-Gal-(1 → 3)-β-Gal-(1 → 4).

type was evident from the occurrence of the M + Na^+ ions at *m/z* 2650 (afuco), 2824 (fuco), 2335 (B-type), and 668 (Hex → Hex → HexNAc). The sequence α-Gal-(1 → 3)-β-Gal-(1 → 4)-β-GlcNAc was deduced from the signal occurring at δ 5.144 [for the α-(1 → 3)-linked Gal residue] and δ 4.542 [for the β-(1 → 4) linked Gal residue][37]. The terminal α-Gal-(1 → 3)-β-Gal sequence could be easily located on the (1 → 6) antenna as only the (1 → 3) branch is fully sialylated. On the other hand, the H-1 resonances of the Gal-*6* and Gal-*6'* residues in this type of structure were found at δ 4.539 and 4.542, respectively. Apparently, the other ^{1}H NMR parameters seem not to be affected by this terminal sequence. Therefore, the glycan of the (1 + 3) antennary type possesses the structure corresponding to oligosaccharide-alditol **4**. The same molecular M + Na^+ ions at *m/z* 2824 (fuco) and 2650 (afuco) arose from the isomeric glycan structure with the (2 + 6)-type antenna (a very minor structure not shown), whose A^+ ions appeared at *m/z* 1029 → 997 (NeuAc → Hex → Hex → $HexNAc^+$) and *m/z* 464 → 432 (Hex → $HexNAc^+$). The glycan structures of the (2 + 4) type, isomeric to the (1 + 5) type,

Type



Scheme 2. Structures of the various oligosaccharide-alditols that may be isolated from Con A-Fraction B. Type 5 was only observed by FABMS.

TABLE III

Various types of antennae present in the oligosaccharide-alditols of Con A-Fraction B, as deduced from the FABMS, fragment ions

Type of antenna	Fragmentations	*m/z*
1	NeuAc → Hex → HexNAc$^+$	825 → 793
2	Hex → HexNAc$^+$	464 → 432
3	Hex → Hex → HexNAc$^+$	668 → 636
4	NeuAc → HexNAc → HexNAc$^+$	866 → 834; 621
5	HexNAc → HexNAc$^+$	505 → 473; 260
6	NeuAc → Hex → Hex → HexNAc$^+$	1029 → 997; 580

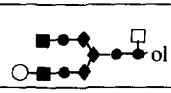
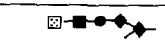
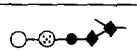
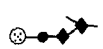
TABLE IV

Various types of combination of the antennae present in the oligosaccharide-alditols of Con A-Fraction B, as deduced from the molecular ions M+Na$^+$ and the ions of type *C*

Combination	*C*$^+$ ion	M+H$^+$	M+Na$^+$	M+H$^+$	M+Na$^+$
		Fuc absent		Fuc present	
1+1	2492	2785	2807	2959	2981
1+2	2131	2424	2446	2598	2620
1+3	2335	2628	2650	2802	2824
1+4	(2533)	2826	2848	3000	3022
1+5	2172	(2465)	(2487)		2661
2+4	2172	(2465)	(2487)		2661
2+2	1770	2063	2085		
3+4	(2376)			2843	2865
2+6	2335	2628	2650	2802	2824

TABLE V

^{1}H Chemical shifts (δ) of the structural reporter group protons for the oligosaccharide-alditols isolated from the bovine lactotransferrin Con A-Fraction B [a]

Report group	Sugar				
H-1	GlcNAc-*2*	4.641 (4.174)			
	Man-*3*	4.777	4.777	4.777	4.777
	Man-*4*	4.136		5.136	5.123
	Man-*4′*	4.928	4.928		
	GlcNAc-*5*	4.605		4.581	4.581
	GlcNAc-*5′*	4.581	4.581		
	Gal-*6*	4.445			
	Gal-*6′*	4.471	4.542		
	GalNAc(GN) [b]			4.498	4.515
	α-Gal-(1 → 3)(G3) [c]		5.144		
	α-Fuc-(1 → 6)	4.896			
H-2	Man-*3*	4.254	4.254	4.254	4.253
	Man-*4*	4.191		4.191	4.191
	Man-*4′*	~ 4.10	~ 4.10		
H-3*a*	α-NeuAc-(2 → 6)	1.717		1.717	
H-3*e*	α-NeuAc-(2 → 6)	2.672		2.669	
H-4	Gal-*6′*		4.182		
H-5	α-Gal-(1 → 3)		4.205		
NAc	GlcNAcol	2.056			
	GlcNAc-*2*	2.080 (2.088)			
	GlcNAc-*5*	2.070		2.070	~ 2.05
	GlcNAc-*5′*	2.047	2.047		
	GalNAc			2.070	2.066
	NeuAc	2.030		2.030	
CH_3	α-Fuc-(1 → 6)	(1.226)			

[a] Compounds are represented by the schematic structures according to Vliegenthart et al.[34]: ⊛, GalNAc; ●, GlcNAc; ◆, Man; ■, β-Gal-(1 → 4); □, Fuc; ○, Neu5Ac; and ⊠, α-Gal-(1 → 3). [b] GN denotes GalNAc in the ^{1}H NMR spectrum (Fig. 2). [c] G3 denotes α-Gal-(1 → 3) in the ^{1}H NMR spectrum (Fig. 2).

were characterized by the ions at m/z 2661 ($M + Na^+$) and m/z at 866 (NeuAc → HexNAc → HexNAc). According to the high intensity of the H-1 signal of GalNAc, at δ 4.498, and the low content of sialyl groups of the (1 → 6) antenna, the α-NeuAc-(2 → 6)-β-GalNAc-(1 → 4)-β-GlcNAc structure could be located in the (1 → 3) antenna. The position of the GalNAc residue in the (1 → 3) antenna was confirmed by desialylation of Fraction B. No difference was observed in the chemical shift of H-1 of Man-*4′* before or after desialylation (namely *4′* and $4'_{as}$); so it was concluded that the (1 → 6)-linked antenna is not sialylated. As the FABMS analysis indicated an α-NeuAc-(2 → 6)-β-GalNAc-(1 → 4)-β-GlcNAc-(1 → 2) structure, this unit is necessarily linked to the (1 → 3) antenna. The high intensity of the singlet observed at δ 2.070 probably includes the resonance of the NAc group of GalNAc, GlcNAc-5 (sialo), and GlcNAc-*5* substituted by GalNAc (see Fig. 2 and Scheme 2). The desialylation of Fraction B led to an intense

TABLE VI

Molar carbohydrate composition [a] of oligosaccharides present in Con A-Fraction C isolated from bovine lactotransferrin

Oligosaccharide-alditols	Man	GlcNAc
C-I	3.6	1.0
C-II	5.3	1.0
C-III	5.7	1.0
C-IV	6.4	1.0
C-V	7.8	1.0
C-VI	8.5	1.0

[a] Molar ratios were calculated on the basis of one 2-acetamido-2-deoxyglucose unit.

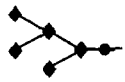
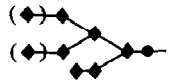
deshielding of all the NAc signals, except for that observed at δ 2.066, which was consequently attributed to GalNAc. These values are similar to those described for the sequence α-NeuAc-(2 → 6)-β-GalNAc-(1 → 4)-β-GlcNAc of human lutropin[38]. According to these results, the oligosaccharide-alditols corresponding to the (2 + 4) antennary type possess structure **7**. The presence of a glycan possessing structure **5** of the (1 + 4) antennary type was deduced from the occurrence of the $M + Na^+$ ion at m/z 3022. The glycan having structure **6** containing the (1 + 5) antennary type did not exceed 5% of the mixture and could only be characterized by the presence of the molecular ion at m/z 2661 together with that of the signal observed at δ 4.514.

Structure determination of Con A-Fraction C oligosaccharides.—Con A-Fraction C, strongly retained on a Con A–Sepharose column, contained glycans of the oligomannose type (Table I). The endo-*N*-acetyl-β-D-glucosaminidase from *Sporotricum dimorphosphorum*[18] is a powerful tool for removing the carbohydrate chains from glycopeptides of the oligomannose type[19], and treatment of Fraction C released oligosaccharides that were separated by HPLC on an Amino AS-5A column. Six fractions (Fractions C-I–C-VI) containing 4–9 residues of mannose were separated and characterized (Table VI). An aliquot of each fraction analyzed by TLC showed that the different fractions were relatively homogeneous. The glycan structures of Fractions C-V and C-VI containing, respectively, 8 and 9 mannose units have been previously determined by methylation and ^1H NMR analysis of the corresponding glycopeptides[14]. The structures of the oligosaccharides fractions (C-I–C-IV) were analyzed by ^1H NMR (Table VII). After reduction with sodium borohydride, the reduced oligosaccharides were methylated and analyzed by GLC–MS (Table II).

In the reduced oligosaccharide C-I containing 3.6 units of mannose for one unit of *N*-acetylglucosaminitol, the presence of a very small amount of 2,4,-di-*O*-methylmannoside suggested the absence of the disubstituted mannosyl residue of the trimannose common core. Moreover, the occurrence of 1.2 residue of 2,3,4,6-tetra-*O*-methylmannoside confirmed the presence of only one mannosyl group in terminal nonreducing position. Thus, the results (Table II) are in agreement with the linear structure **8** (Scheme 3). The structure of the nonreduced oligosaccharide was

TABLE VII

^{1}H Chemical shifts (δ) of the structural reporter group protons for oligosaccharides isolated from bovine lactotransferrin Con A-Fraction C [a]

Reporter group	Sugar	Con A-Fraction C					
		C-I	C-II	C-III		C-IV	
							
				A [b]	B [b]	A [b]	B [b]
H-1	GlcNAc-2α	5.205	5.246	5.246		5.246 (with Man-*D2*)	5.228
	β	4.700	4.706	4.710		4.710	
NAc	GlcNAc-2α	2.042	2.043	2.043		2.042	
	β	2.040				[c]	
H-1	Man-*3*	4.772	4.780	4.780		4.770	
	Man-*4*	5.345	5.101	5.348 (with Man-*C*)		5.343	5.350
	Man-*4'*		4.872	4.871		4.872	
	Man-*A*α		5.078	5.076	5.397	5.081	5.399
	Man-*A*β		5.105	5.102		5.405	5.104
	Man-*B*		4.907	4.907	5.142	4.909	5.144
	Man-*C*	5.298		5.052		5.055	5.301
	Man-*D1*	5.042					5.041
	Man-*D2*				5.052		5.055
	Man-*D3*				5.038		5.041
H-2	Man-3α	4.221	4.265	4.253		4.238	
	β	4.208	4.251	4.253		4.228	
	Man-*4*	4.082	4.077	n.d.		4.130	
	Man-*4'*α		4.142	4.144		4.156	
	β		4.148			4.151	
	Man-*A*α		4.048	[c]	4.059	4.088	
	β		4.063			[c]	[c]
	Man-*B*		3.980	3.985	4.020	3.981	4.020
	Man-*C*	4.104		4.068		4.071	4.106
	Man-*D1*	4.064				4.071	
	Man-*D2*			4.068		4.071	
	Man-*D3*			4.068		4.071	

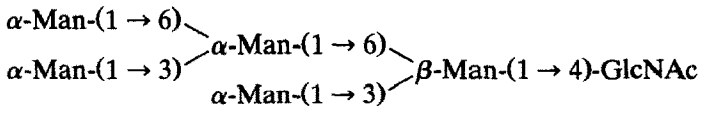
[a] Compounds are represented by the schematic structures according to Vliegenthart et al.[34]; ●, GlcNAc; ◆, Man. [b] A, without Man-*D* residues; B, with Man-*D* residues. [c] Not determined.

confirmed by ^{1}H NMR spectroscopy, as the chemical shifts of the structural reporter groups (Table VII) are essentially identical to those reported for the same glycan structure previously described[39].

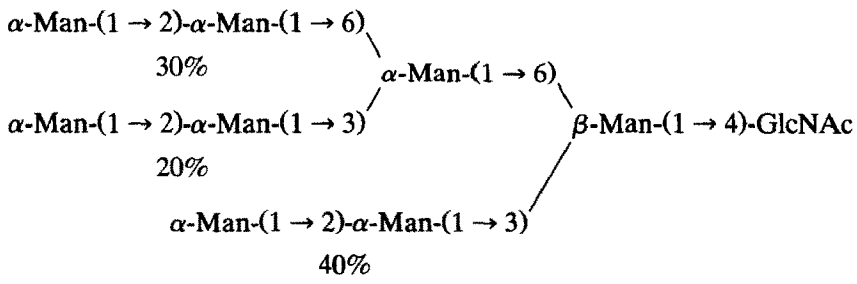
In the reduced oligosaccharide C-II containing 5.3 units of mannose for one unit of *N*-acetylglucosaminitol, the presence of 2.5 residues of 2,3,4,6-tetra-*O*-methylmannoside (Table II) indicated that 2–3 mannose units are in the external position, and the presence of 2 residues of 2,4-di-*O*-methylmannoside indicated that the structure contains two branching points. The ^{1}H NMR spectroscopic data of the nonreduced oligosaccharide C-II (Table VII) matched those of reference

α-Man-(1 → 2)-α-Man-(1 → 2)-α-Man-(1 → 3)-β-Man-(1 → 4)-GlcNAc

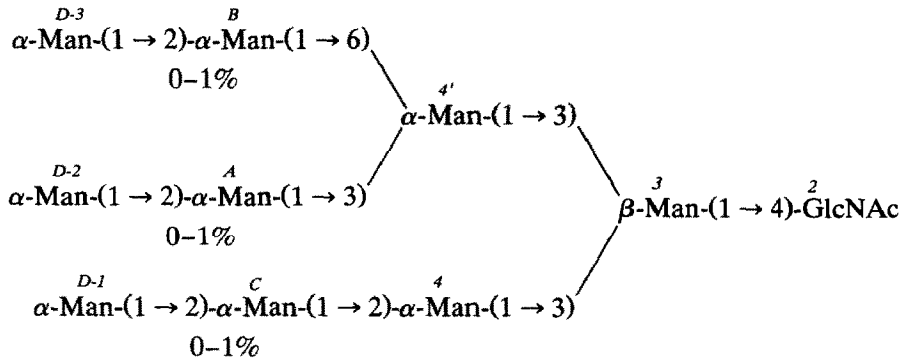
8 (Oligosaccharide C-I)



9 (Oligosaccharide C-II)



10 (Oligosaccharide C-III)



11 (Oligosaccharide C-IV)

Scheme 3. Structures of oligosaccharides isolated from glycopeptides Con A-Fraction C of bovine lactotransferrin as determined by ^{1}H NMR spectroscopy.

compound U7.2, described recently[40], and together these results identified the structure of oligosaccharide C-II as **9**.

In the reduced Fraction C-III containing 5.7 units of mannose for one of *N*-acetylglucosaminitol, 2 residues of 2,4-di-*O*-methylmannoside and 2.5 residues of 2,3,4,6-tetra-*O*-methylmannoside were identified, as in the reduced oligosaccharide C-II. The reduced fraction C-III differed, however, from the reduced oligosaccharide C-II by the presence of 1 residue of 3,4,6-tri-*O*-methylmannoside. The ^{1}H NMR spectrum of the nonreduced fraction C-III indicated that it consists of a

mixture of several compounds, the basic structure being $Man_5GlcNAc$ with a heterogeneity in the terminal α-(1 → 2)-linked mannosyl groups (**10**).

In the reduced fraction C-IV containing 6.4 units of mannose for 1 unit of *N*-acetylglucosaminitol, the presence of 1.9 residues of 3,4,6-tri-*O*-methylmannoside and 2.7 residues of 2,3,4,6-tetra-*O*-methyl mannoside for 2 residues of 2,4-di-*O*-methylmannoside (Table II) confirmed the structure $Man_7GlcNAc$ (**10**). Moreover, the presence of different isomers of $Man_7GlcNAc$ could be deduced from the results obtained by ^1H NMR analysis (Table VII) showing that Man-*A*, Man-*B*, and Man-*C* (units of **11**) are or are not substituted by Man-*D2*, Man-*D3*, and Man-*D1* groups, respectively.

DISCUSSION

Several structures of glycans of different serotransferrins and lactotransferrins have been previously determined[15,16]. The results obtained have shown that the glycan structures are species specific and that the structural diversity of the glycans, so far analyzed, appears to be linked either to the number of antennae or to the peripheral sugar sequences. In fact, the serotransferrin from normal human serum contains diantennary glycans disialylated with *N*-acetyl-α-neuraminic acid residues (2 → 6) linked and two types of triantennary glycans with a group of *N*-acetylneuraminic acid (2 → 3) linked to the third antenna[41–43]. The mouse serotransferrin diantennary glycans are sialylated either by *N*-acetyl- or *N*-glycolyl-neuraminic acid residues[44], the horse serotransferrin diantennary glycans are sialylated by one or two *O*-acetyl-*N*-acetylneuraminic acid groups[45], and the rat serotransferrin diantennary glycans are trisialylated with a third *N*-acetyl-α-neuraminic acid (2 → 6) linked to the peripheral *N*-acetylglucosamine residue[46]. The diantennary human lactotransferrin glycans appeared to be partially sialylated and mono- or di-fucosylated[36]. Bovine lactotransferrin represents, therefore, the first type of glycoprotein of the transferrin family containing both *N*-acetyllactosamine and oligomannoside-type glycans. The different structures of oligomannoside glycans, Man_5–Man_9, are related to the different steps of biosynthesis of the *N*-acetyllactosamine type glycans[47–49]. This observation suggests either that the transport of bovine lactotransferrin through the Golgi apparatus is very rapid and does not allow the complete trimming and glycosylation of all the glycans, or that the folding of the protein inhibits the activity of the glycosyltransferases involved in the biosynthesis of the *N*-acetyllactosamine-type glycans located at specific glycosylation sites. The nonreduced, linear oligosaccharide C-1 (**8**) has been previously characterized in mannosidosis urine[50–52]. The occurrence of this linear pentasaccharide, which is not produced during the catabolic processing pathway, may be explained by the existence of an endo-α-D-mannosidase that cleaves specifically the α-Man-(1 → 6)-β-Man linkage.

The heterogeneity of the structure of the *N*-acetyllactosamine-type glycans of bovine lactotransferrin was resolved by use of mass spectrometry and ^1H NMR

spectroscopy. Common diantennary *N*-acetyllactosamine glycans, partially sialylated and fucosylated, were characterized in association with some particular diantennary structures that carry α-Gal-(1 → 3)-β-Gal, β-GalNAc-(1 → 4)-β-GlcNAc, or α-NeuAc-(2 → 6)-β-GalNAc-(1 → 4)-β-GlcNAc units. The structural element α-Gal-(1 → 3)-β-Gal-(1 → 4)-β-GlcNAc was reported to occur in the carbohydrate chains of other bovine glycoproteins, such as bovine thyroglobulin[37,53,54]. Analysis of the acceptor specificity of the (1 → 3)-α-D-galactosyltransferase purified from calf serum has demonstrated that the (1 → 3)-α-D-galactosyltransferase competes with the (2 → 6)- and (2 → 3)-α-sialyltransferases for the *N*-acetyllactosamine units in a mutually exclusive way[55]. In our study, the α-Gal-(1 → 3)-β-Gal unit was found to be located on the antenna linked to the α-Man-(1 → 6) residue. Similar results were obtained for the glycoprotein of Friend murine leukemia virus[56], whereas in the glycopeptides derived from a Lewis lung carcinoma cell subline, resistant to *Aleuria aurantia* agglutinin, the α-Gal-(1 → 3) unit was found linked to both antennae of a diantennary glycan[57].

2-Acetamido-2-deoxy-D-galactose has not been found in the structure of transferrin glycans so far analyzed. The presence of this monosaccharide unit in *N*-glycosyl-linked glycans appears to be limited. In some glycoproteins, the D-galactosyl residue of the *N*-acetyllactosamine unit may be replaced by a 2-acetamido-2-deoxy-β-D-galactosyl residues (1 → 4) linked to the peripheral 2-acetamido-2-deoxy-D-glucosyl residue. Terminal, β-linked 2-acetamido-2-deoxygalactosyl residues were previously identified in the glycans of glycoproteins synthesized by *Schistosoma mansoni* adult males[58]. In some glycoproteins, substituted 2-acetamido-2-deoxy-β-D-galactosyl residues were found. For instance, the following units, → 3)-*O*-Gal-(1 → 3)-β-GalNAc-(1 → 4)-β-GlcNAc-(1 → 2)-α-Man-(1 → and α-Fuc-(1 → 2)-β-Gal-(1 → 3)-β-GalNAc-(1 → 4)-β-GlcNAc-(1 → 2)-α-Man-(1 → , were observed in glycans derived from hemocyanin of *Lymnaea stagnalis*[59]. The terminal sequence β-GalNAc(SO_3^-)-(1 → 4)-β-GlcNAc-(1 → 2)-α-Man has been observed in glycoprotein hormones synthesized by the anterior pituitary gland from several species[60–64], as well as in one or in both antennae of a diantennary, *N*-acetyllactosamine-type glycan or in the (1 → 3) linked antenna of a hybrid-type glycan[65]. Moreover, in human lutropin[38], the sialyl group-containing terminal sequence, α-NeuAc-(2 → 6)-β-GalNAc-(1 → 4)-β-GlcNAc, was recently characterized in the (1 → 3) linked antenna. In the present work, the 2-acetamido-2-deoxygalactose unit was found in bovine lactotransferrin glycans, either nonsubstituted or substituted by a α-NeuAc-(2 → 6) residue in the (1 → 3) linked antenna. These results are in agreement with the results obtained by Weisshaar et al.[38] on human lutropin, but in disagreement with our preliminary data[16,66]; this suggests the presence, in bovine mammary gland, of an *N*-acetyl-D-galactosaminyltransferase that recognizes preferentially the 2-acetamido-2-deoxy-D-glucose unit (1 → 2) linked to the α-Man-(1 → 3) residue. Preliminary results[67] of the analysis of the structure of the glycans linked at individual glycosylation site in bovine lactotransferrin showed that the diantennary glycans containing the [α-

NeuAc-(2 → 6)]$_{0-1}$-β-GalNAc-(1 → 4)-β-GlcNAc unit are located only at Asn 490. This observation suggests that the activity of the *N*-acetylgalactosaminyltransferase from bovine mammary gland, like the *N*-acetylgalactosaminyltransferase from the pituitary glands[68,69], is influenced by the surrounding polypeptide chain at the glycosylation site. Further analysis of the structural features of the peptide responsible for the specificity of the *N*-acetylgalactosaminyltransferase from bovine mammary gland should bring complementary results concerning the specificity of the *N*-acetylgalactosaminyltransferases.

ACKNOWLEDGMENTS

This work was supported, in part, by the Centre National de la Recherche Scientifique (Unité Mixte de Recherche du CNRS No. 111, Relations Structure–Fonction des Constituants Membranaires; Director, Professor A. Verbert), by the Université des Sciences et Techniques de Lille Flandres–Artois, by the Association pour la Recherche sur le Cancer, and by the Institut National de la Santé et de la Recherche Médicale (Contrat No. 882016). The authors thank Mrs. A. Honvault and Mr. Y. Leroy, CNRS technicians, for their skilful technical assistance. They are grateful to the Conseil Régional de la Région Nord–Pas de Calais, the Centre National de la Recherche Scientifique, the Ministère de l'Éducation Nationale, and the Association de la Recherche sur le Cancer for their contribution to the acquisition of the 400-MHz ^{1}H NMR spectrometer.

REFERENCES

1 J. Montreuil and S. Mullet, *C.R. Hebd. Séances Acad. Sci., Ser. D*, 250 (1960) 176–178; J. Montreuil, J. Tonnelat, and S. Mullet, *Biochim. Biophys. Acta*, 45 (1960) 413–421.
2 B. Blanc and H. Islisker, *Bull. Soc. Chim. Biol.*, 43 (1961) 929–935.
3 P.L. Masson, *La Lactoferrine, Protéine des Sécrétions Externes et des Leucocytes Neutrophiles*, Arsca, Brussells, 1970.
4 M.L. Groves, *J. Am. Chem. Soc.*, 82 (1960) 3345–3350.
5 P.E. Mead and J.W. Tweedie, *Nucl. Acids Res.*, 18 (1990) 7167.
6 A. Pierce, D. Colavizza, M. Benaissa, P. Maes, A. Tartar, J. Montreuil, and G. Spik, *Eur. J. Biochem.*, 196 (1991) 177–184.
7 M.H. Metz-Boutigue, J. Jollès, J. Mazurier, F. Schoentgen, D. Legrand, G. Spik, J. Montreuil, and P. Jollès, *Eur. J. Biochem.*, 145 (1984) 659–676.
8 N.J. Powell and J.E. Ogden, *Nucl. Acids Res.*, 18 (1990) 4013; M.W. Rey, S.L. Woloshuk, H.A. de Boer and F.R. Pieper, *ibid.*, 18 (1990) 5288.
9 B.T. Pentecost and C.T. Teng, *J. Biol. Chem.*, 262 (1987) 10134–10139.
10 P. Aisen and I. Listowsky, *Annu. Rev. Biochem.*, 49 (1980) 357–393.
11 J. Montreuil, J. Mazurier, D. Legrand, and G. Spik, in G. Spik, J. Montreuil, R.R. Crichton, and J. Mazurier (Eds.), *Proteins of Iron Storage and Transport*, Elsevier, Amsterdam, 1985, pp 25–38.
12 B. Gorinsky, C.R.L. Horsburg, P.F. Lindley, D.S. Moss, M. Parkar, and J.L. Watson, *Nature (London)*, 281 (1979) 157–158.
13 F.J. Castellino, W.W. Fish, and K.G. Mann, *J. Biol. Chem.*, 245 (1970) 4269–4275.
14 H. van Halbeek, L. Dorland, J.F.G. Vliegenthart, G. Spik, A. Chéron, and J. Montreuil, *Biochim. Biophys. Acta*, 675 (1981) 293–296.

15 G. Spik, B. Coddeville, D. Legrand, J. Mazurier, D. Léger, M. Goavec, and J. Montreuil, in G. Spik, J. Montreuil, R.R. Crichton, and J. Mazurier (Eds.), *Proteins of Iron Storage and Transport*, Elsevier, Amsterdam, 1985, pp 47–51.
16 G. Spik, B. Coddeville, and J. Montreuil, *Biochimie*, 70 (1988) 1459–1469.
17 G. Strecker and J. Montreuil, *Biochimie*, 61 (1979) 1199–1246.
18 S. Bouquelet, G. Strecker, J. Montreuil, and G. Spik, *Biochimie*, 62 (1980) 43–49.
19 O. Kol, C. Brassard, G. Spik, J. Montreuil, and S. Bouquelet, *Glycoconjugate J.*, 6 (1989) 333–348.
20 J.F.G. Vliegenthart, L. Dorland, and H. van Halbeek, *Adv. Carbohydr. Chem. Biochem.*, 41 (1983) 209–374.
21 H. Egge and J. Peter-Katalinic, *Mass Spectrom. Rev.*, 6 (1987) 331–393.
22 J. Montreuil, S. Bouquelet, H. Debray, B. Fournet, G. Spik, and G. Strecker, in M.F. Chaplin and J.F. Kennedy (Eds.), *Carbohydrate Analysis: a Practical Approach*, IRL Press, Oxford, 1987, pp 143–204.
23 J.C. Zanetta, W.C. Breckenridge, and G. Vincendon, *J. Chromatogr.*, 69 (1972) 291–304.
24 J.P. Kamerling, G.J. Gerwig, J.F.G. Vliegenthart, and J.R. Clamp, *Biochem. J.*, 151 (1975) 491–495.
25 S. Hakomori, *J. Biochem. (Tokyo)*, 55 (1964) 205–208.
26 J. Paz-Parente, P. Cardon, Y. Leroy, J. Montreuil, B. Fournet, and G. Ricard, *Carbohydr. Res.*, 141 (1985) 41–47.
27 B. Fournet, G. Strecker, Y. Leroy, and J. Montreuil, *Anal. Biochem.*, 116 (1981) 489–502.
28 A. Chéron, J. Mazurier, and B. Fournet, *C.R. Hebd. Séances Acad. Sci., Ser. D*, 284 (1977) 585–588.
29 A.M. Crestfield, S. Moore, and W.H. Stein, *J. Biol. Chem.*, 238 (1963) 622–627.
30 G. Spik and J. Montreuil, *Bull. Soc. Chim. Biol.*, 51 (1969) 1271–1285.
31 T. Krusius, J. Finne, and H. Rauvala, *FEBS Lett.*, 71 (1976) 117–120.
32 D.M. Carlson, *J. Biol. Chem.*, 243 (1968) 616–626.
33 Y.C. Lee and J.R. Scocca, *J. Biol. Chem.*, 247 (1978) 5733–5758.
34 C.L. Reading, E. Penhoet, and C. Ballow, *J. Biol. Chem.*, 253 (1978) 5600–5612.
35 S.J. Turco, *Anal. Biochem.*, 118 (1981) 278–286.
36 G. Spik, G. Strecker, B. Fournet, S. Bouquelet, J. Montreuil, L. Dorland, H. van Halbeek, and J.F.G. Vliegenthart, *Eur. J. Biochem.*, 121 (1982) 413–419.
37 L. Dorland, H. van Halbeek, and J.F.G. Vliegenthart, *Biochem. Biophys. Res. Commun.*, 22 (1984) 859–866.
38 G. Weisshaar, J. Hiyama, A.G.C. Renwick, and M. Nimtz, *Eur. J. Biochem.*, 195 (1991) 257–268.
39 H. van Halbeek, L. Dorland, G.A. Veldink, J.F.G. Vliegenthart, G. Strecker, J.M. Michalski, J. Montreuil, and W.E. Hull, *FEBS Lett.*, 121 (1980) 71–77.
40 K. Hard, A. Mekking, J.P. Kamerling, G.A.A. Dacremont, and J.F.G. Vliegenthart, *Glyconjugate J.*, 8 (1991) 17–28.
41 G. Spik, B. Bayard, B. Fournet, G. Strecker, S. Bouquelet, and J. Montreuil, *FEBS Lett.*, 50 (1975) 296–296.
42 L. Dorland, J. Haverkamp, B.L. Schut, J.F.G. Vliegenthart, G. Spik, G. Strecker, B. Fournet, and J. Montreuil, *FEBS Lett.*, 77 (1977) 15–20.
43 G. Spik, V. Debruyne, J. Montreuil, H. van Halbeek, and J.F.G. Vliegenthart, *FEBS Lett.*, 183 (1985) 65–69.
44 Y. Leclercq, G. Sawatzki, J.M. Wieruszeski, J. Montreuil, and G. Spik, *Biochem. J.*, 247 (1987) 571–578; B. Coddeville, unpublished results.
45 B. Coddeville, A. Stratil, J.M. Wieruszeski, G. Strecker, J. Montreuil, and G. Spik, *Eur. J. Biochem.*, 186 (1989) 583–590.
46 G. Spik, B. Coddeville, G. Strecker, J. Montreuil., E. Regoeczi, P.A. Chindemi, and J.R. Rudolph, *Eur. J. Biochem.*, 195 (1991) 397–405.
47 J. Montreuil, *Adv. Carbohydr. Chem. Biochem.*, 37 (1980) 157–223.
48 J. Montreuil, in A. Neuberger and L.L.M. van Deenen (Eds.), *Comprehensive Biochemistry*, Vol. 19B, Part II, Elsevier, Amsterdam, 1982, pp 1–188.
49 R. Kornfeld and S. Kornfeld, *Annu. Rev. Biochem.*, 54 (1985) 631–664.
50 N.E. Nordan, A. Lundblad, S. Swensson, and S. Autio, *Biochemistry*, 13 (1974) 871–874.
51 K. Yamashita, Y. Tachibana, K. Mihara, S. Okada, H. Yabuuchi, and A. Kobata, *J. Biol. Chem.*, 255 (1980) 5126–5133.

52 H. van Halbeek, L. Dorland, G.A. Velding, J.F.G. Vliegenthart, G. Strecker, J.C. Michalski, J. Montreuil, and W.E. Hull, *FEBS Lett.*, 121 (1980) 71–77.
53 R.D. Cummings and S. Kornfeld, *J. Biol. Chem.*, 257 (1982) 11230–11234.
54 R.G. Spiro and V.D. Bhoyroo, *J. Biol. Chem.*, 259 (1984) 9858–9866.
55 W.M. Blanken and D.H. van den Eijden, *J. Biol. Chem.*, 260 (1985) 12927–12934.
56 R. Geyer, H. Geyer, S. Stirm, G. Hunsmann, J. Schneider, U. Dabrowski, and J. Dabrowski, *Biochemistry*, 23 (1984) 5628–5637.
57 H. Debray, D. Dus, J.M. Wieruszeski, G. Strecker, and J. Montreuil, *Glycoconjugate J.*, 8 (1991) 29–37.
58 K. Nyame, D.F. Smith, R.T. Damian, and R.D. Cummings, *J. Biol. Chem.*, 264 (1989) 3235–3243.
59 J.A. Van Kuik, R.T. Sijbesma, J.P. Kamerling, J.F.G. Vliegenthart, and E.J. Wood, *Eur. J. Biochem.*, 169 (1987) 399–411.
60 O.P. Bahl, M.S. Reddy, and G.S. Bedi, *Biochem. Biophys. Res. Commun.*, 96 (1980) 1192–1199.
61 E.D. Green, H. van Halbeek, I. Boime, and J.U. Baenziger, *J. Biol. Chem.*, 260 (1985) 15623–15630.
62 E.D. Green, J.U. Baenziger, and I. Boime, *J. Biol. Chem.*, 260 (1985) 15631–15638.
63 E.D. Green and J.U. Baenziger, *J. Biol. Chem.*, 263 (1988) 25–35.
64 J.U. Baenziger and E.D. Green, *Biochim. Biophys. Acta*, 947 (1988) 287–306.
65 E.D. Green, I. Boime, and J.U. Baenziger, *J. Biol. Chem.*, 261 (1986) 16309–16316.
66 G. Spik, B. Coddeville, Y. Leclercq, J.M. Wieruszeski, H. Egge, and J. Montreuil, *Proc. Int. Symp. Glycoconjugates, IVth*, (1987) Abstr. A 105.
67 D. Colavizza, personal communication.
68 P.L. Smith and J.U. Baenzinger, *Science*, 242 (1988) 930–932.
69 P.L. Smith and J.U. Baenzinger, *Proc. Natl. Acad. Sci. U.S.A.*, 87 (1990) 7275–7279.