Eur. J. Biochem. *139*, 337–349 (1984)

Primary structure of *N*-glycosidically linked asialoglycans of secretory immunoglobulins A from human milk

Annick PIERCE-CRETEL, Henri DEBRAY, Jean MONTREUIL, and Geneviève SPIK

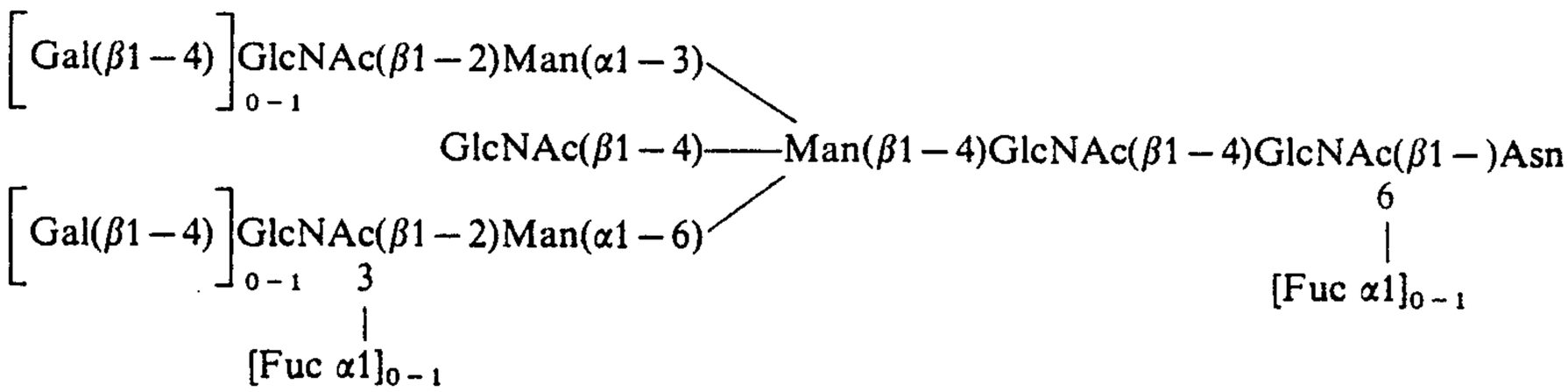
Laboratoire de Chimie Biologique et Laboratoire Associé au Centre National de la Recherche Scientifique no 217, Université des Sciences et Techniques de Lille I

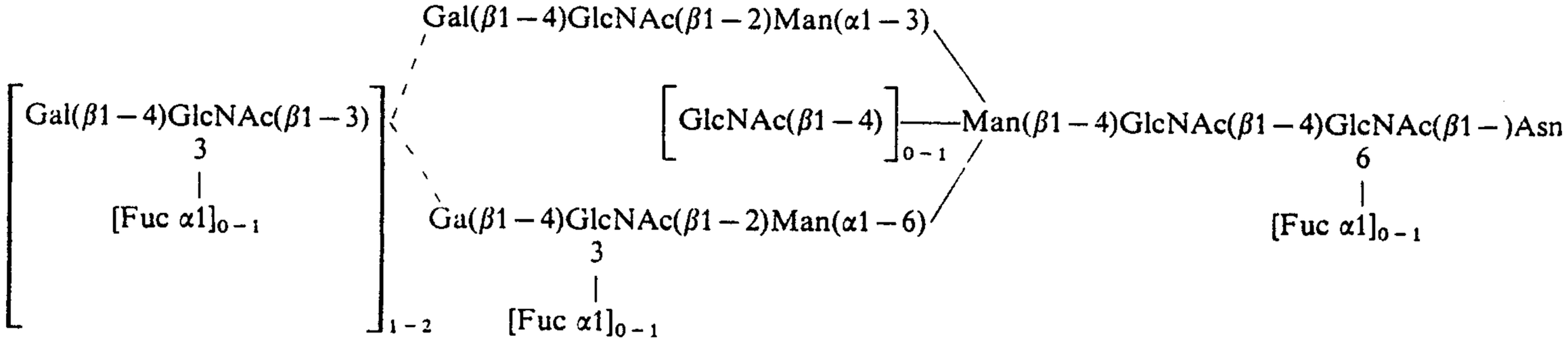
Herman VAN HALBEEK, Johanna H. G. M. MUTSAERS, and Johannes F. G. VLIEGENTHART

Department of Bio-Organic Chemistry, University of Utrecht

(Received June 21/October 4, 1983) – EJB 83 0653

The asialoglycopeptides obtained from secretory immunoglobulins A from human milk have been separated by gel filtration and affinity chromatography on Concanavalin A-Sepharose and *Lens culinaris* agglutinin-Sepharose columns. Their structures have been determined by sugar analysis, methylation studies including mass spectrometry and 500-MHz ^{1}H-NMR spectroscopy. The glycans are of the biantennary *N*-acetyllactosamine type differing in their degree of extension by fucosyl-*N*-acetyllactosamine residues. The overall structures of the glycopeptides are as follows:





Most of the asialoglycopeptide structures possess an intersecting GlcNAc residue; they are suggested to be located on the α chain of the secretory immunoglobulins A of human milk. The non-intersected structures probably occur on the secretory piece. The methodology applied to the structural analysis adequately coped with the extremely high degree of heterogeneity shown by the structures.

Human milk secretory immunoglobulins A (sIgA) present an exceptional diversity in the structure of the sugar moieties attached to the polypeptide chains. *O*-Glycosidically linked glycans are present at the hinge region of the α chains, the structures of which have been described previously [1, 2]. The *N*-glycosically-linked glycans present on the α-chains and the junction and secretory pieces [3–5] can be divided into two categories according to the presence or absence of sialic acid residues. The structures of the sialic acid-containing glycans (acidic fraction) were reported in a previous paper [6]. The neutral fraction is extremely heterogeneous necessitating the use of lectin affinity chromatography [7, 8] to achieve the separation of the different glycan structures. In this paper we describe the separation of the *N*-glycosidic glycans present in the neutral fraction and their structural analysis. The latter was performed by methylation analysis, mass spectrometry and high-resolution 500MHz ^{1}H-NMR sprectroscopy.

Abbreviations. Fuc, L-fucose; Gal, D-galactose, Man, D-mannose; GlcNAc, *N*-acetyl-D-glucosamine, Asn, L-asparagine; ConA, concanavalin A; sIgA secretory immunoglobulins A; WEFT, water elimination Fourier transform.

Enzyme. Endo-*N*-acetyl-β-D-glucosaminidase D (EC 3.2.1.96).

MATERIALS AND METHODS

Methyl α-D-glucopyranoside was purchased from Koch-Light Laboratories Ltd (Colnbrook, England). Endo-*N*-acetyl-β-D-glucosaminidase D from *Diplococcus pneumoniae* was from Seikagaku Kogyo Co. Ltd (Tokyo, Japan). Silicagel thin-layer chromatography plates (Kieselgel 60 F 254) were

from Merck (Darmstadt, FRG). Bio-Gel P-30 (200–400 mesh) and Bio-Gel P-2 (200–400 mesh) were from Bio-Rad Laboratories (Richmond, CA, USA). ^2H_2O was from Aldrich (Milwaukee, WI, USA). Sepharose 4B was obtained from Pharmacia Fine Chemicals A. B. (Uppsala, Sweden). *Lens culinaris* agglutinin (Pharmindustrie, Clichy, France) was purified by affinity chromatography [9] and immobilized on activated Sepharose 4B according to March et al. [10] at a concentration of 5 mg of lectin/ml of gel [7].

Preparation of the asialoglycopeptides

Human milk sIgA were purified to 99.3% [1]. They contained a small amount of IgG (less than 0.1%; w/w) and IgM (about 0.6%; w/w). They were successively hydrolyzed by trypsin, pepsin and pronase. Subsequently the neutral (GP-N) and acidic (GP-A) fractions were separated by ion-exchange chromatography [6]. The asialoglycopeptides (GP-N) were fractionated on a Bio-Gel P-30 column (2 × 120 cm) equilibrated in 0.1 M acetic acid at a flow rate of 7.5 ml/h. The carbohydrate fractions were visualized by a phenol/sulfuric acid reagent [11]. The major glycopeptide fraction IV was *N*-[^{14}C]acetylated according to Koide et al. [12] and chromatographed on an immobilized ConA-Sepharose column. After dissolution in 5 mM sodium acetate buffer pH 5.2 containing 0.1 M NaCl and $CaCl_2$, $MnCl_2$, $MgCl_2$ (1 mM each), fraction IV (10 mg) was applied to a column of ConA-Sepharose (1.7 × 20 cm). Elution was carried out first with buffer and then with buffer containing 10 mM methyl α-D-glucopyranoside. Fractions of 1 ml were collected and samples of 5 µl analyzed for radioactivity. Each of the ConA fractions was chromatographed on an immobilized *L. culinaris* agglutinin (1 × 12 cm) column [8] equilibrated in 0.17 M NaCl/0.01 M sodium phosphate buffer pH 7.2. Elution was carried out first with the above buffer and then with this buffer containing 0.15 M methyl α-D-glucopyranoside. Fractions of 500 µl were collected and samples of 5 µl analyzed for radioactivity. All the obtained fractions were desalted on a Bio-Gel P-2 column (1.2 × 46 cm) equilibrated in 0.1 M acetic acid at a flow rate of 4.5 ml/h (750 µl/fraction).

Determination of the structure of the asialoglycopeptides

The molar carbohydrate content of each fraction was determined by gas-liquid chromatography after methanolysis and trifluoroacetylation [13]. Methylation analysis was done according to Finne et al. [14] as modified by Paz Parente et al. [15] allowing the methylation of microquantities. The methyl derivatives were identified after gas-liquid chromatography–mass spectrometry analysis according to Fournet et al. [16]. Hydrolysis of the glycopeptide fraction I by endo-*N*-acetyl-β-D-glucosaminidase D was performed according to Tai et al. [17]. The detection of hydrolysed compounds was previously described [18]. The determination of Lewis^b activity was performed by haemagglutination inhibition as follows: total human non-diluted serum (0.1 ml) titer 8 in saline was incubated for 2 h at room temperature with 0.1 ml of serial twofold dilutions of a solution of compound VI (1 mg/ml). A second incubation was then performed after addition of 0.1 ml human red blood cells of group 0 phenotype Le (a^-b^+) (3% in NaCl 0.9%) for 1 h at 22 °C.

Before NMR analysis, the asialoglycopeptides were treated repeatedly with ^2H_2O (99.96 mol % ^2H) with intermediate lyophilization. The 500-MHz ^{1}H-NMR spectra were recorded on a Bruker WM-500 spectrometer (SON hf-NMR facility, Department of Biophysics, Nijmegen University, The Netherlands) operating in the Fourier transform mode and equipped with a Bruker Aspect-2000 computer. For experimental details see [19, 20]. The probe temperature was 27 °C. Solvent peak suppression for dilute samples was achieved by a water elimination Fourier transform (WEFT) pulse sequence (composite, non-selective 180° pulse-delay τ – 90° pulse-acquisition) [21]. Chemical shifts (δ) are expressed in ppm downfield from internal sodium 4,4-dimethyl-4-silapentane-1-sulfonate but were actually measured by reference to internal acetone (δ 2.225 ppm in ^2H_2O, 27 °C) with an accuracy of 0.002 ppm.

RESULTS

Preparation of the asialoglycopeptides

The trypsin, pepsin and pronase hydrolysis of 1 g of sIgA led to the isolation of 17.5 mg of the neutral fraction GP-N [6]. The amino acid composition of fraction GP-N showed the presence of one aspartic acid residue for 4.6 *N*-acetylglucosamine residues. Threonine, serine, glutamic acid, glycine and valine residues were characterized in trace amount. After Bio-Gel P-30 chromatography of fraction GP-N, seven glycopeptide fractions (I–VII) were obtained. Their relative abundances are given in Table 1. The major fraction (fraction IV) was *N*-[^{14}C]acetylated and subfractionated by lectin affinity chromatography (Fig. 1). Fraction IV was resolved on immobilized ConA-Sepharose into three peaks. The first two were eluted with the starting buffer while the third peak was eluted with 0.01 M methyl α-D-glucopyranoside.

Subsequently each subfraction was submitted to immobilized *Lens culinaris* agglutinin chromatography (Fig. 1). The first retarded ConA fraction was separated into non-retained (IV-1) and retained (IV-2) glycopeptide fractions. The *L. culinaris* agglutinin chromatography of the ConA-retained fraction led to two subfractions IV-4 (non-retained) and IV-5 (retained). No improved separation was obtained from glycopeptide IV-3 which was non-retained on *L. culinaris* agglutinin. The relative abundances of fractions IV-1 to IV-5 have been listed in Table 2.

Primary structure of the asialoglycopeptides

The primary structure of the glycopeptides present in GP-N fractions I to VII was completely established applying a combination of methanolysis, methylation-mass spectrometry and 500-MHz ^{1}H-NMR spectroscopic analysis.

Methanolysis

The carbohydrate compositions of the GP-N glycopeptide fractions I to VII are given in Table 1 and those of subfractions IV-1 and IV-5 in Table 2. Since in most of the fractions the ratios of the monosaccharides, particularly Fuc and/or Gal to Man, are not integers, it is apparent that these fractions are still heterogeneous. The majority of the eleven studied fractions are characterized by the presence of five GlcNAc residues (except VI and VII); they differ from each other with respect to their Fuc and Gal content implying that these glycans possess incomplete structures. The fractions V, VI and VII containing the larger glycopeptides are characterized by relatively large amounts of GlcNAc (5–7), Gal (3–4) and Fuc (2–3.5) residues. They may be considered as extended biantennary *N*-acetyllactosamine structures.

Table 1. *Relative abundance and molar carbohydrate composition of the asialoglycopeptide fractions isolated from human milk sIgA*
The molar ratios were calculated on the basis of three mannose residues

Asialoglycopeptide fraction	Yield	Molar ratio of			
		Fuc	Gal	Man	GlcNAc
	%				
GP-N	–	0.8	1.65	3	4.98
I	15	0.29	0.58	3	3.07
II	9	0.16	0.49	3	4.29
III	11	0.40	0.43	3	4.88
IV	38	0.80	1.05	3	4.98
V	13	2.20	3.00	3	4.87
VI	5	3.50	3.93	3	5.40
VII	9	3.07	4.83	3	6.84

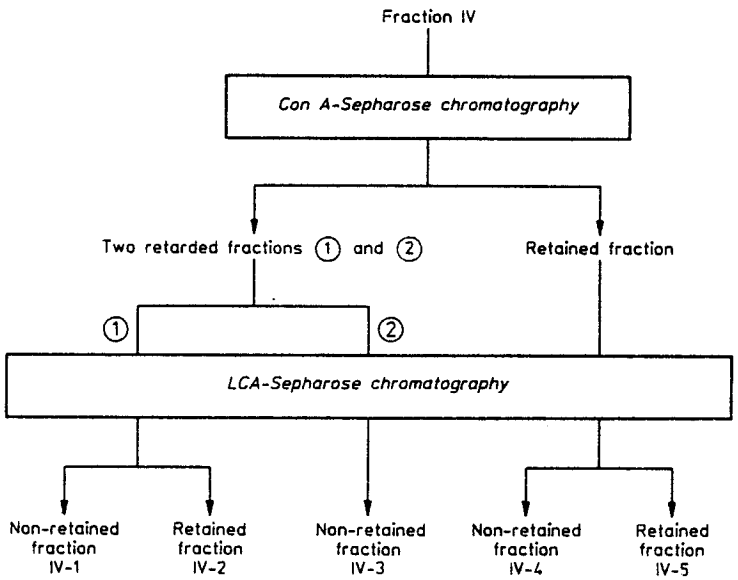


Fig. 1. *Subfractionation of GP-N fraction IV from human milk sIgA by affinity chromatography.* Affinity chromatography was on ConA-Sepharose and Sepharose linked to *Lens culinaris* agglutinin (LCA). For details see Materials and Methods

Table 2. *Relative abundance and molar carbohydrate composition of the asialoglycopeptide subfractions isolated from GP-N fraction IV after affinity chromatography*
Affinity chromatography was on ConA-Sepharose and Sepharose linked to *Lens culinaris* agglutinin. The molar ratios were calculated on the basis of three mannose residues

Asialoglycopeptide subfraction	Yield	Molar ratio of			
		Fuc	Gal	Man	GlcNAc
	%				
IV	–	0.80	1.05	3	4.98
IV-1	0.6	0	1.12	3	4.95
IV-2	7	0.92	1.01	3	5.07
IV-3	0.9	0.18	1.08	3	4.91
IV-4	43.5	0.13	0.86	3	4.89
IV-5	48	1.47	1.04	3	4.98

Methylation analysis

The molar ratios of the different methylated derivatives of the monosaccharides present in the GP-N glycopeptide fractions are described in Table 3. The identification and the quantification of 3,4,6-Me_3Man and 2,4-Me_2Man or 2-Me_1-Man as the only methylated derivatives of mannose led to the conclusion that all GP-N fractions possessed a biantennary type of branching. The presence of 2-Me_1Man instead of 2,4-Me_2Man in glycans I, II, III, IV and V in conjunction with that of permethylated GlcNAc indicated that an intersecting GlcNAc was present in these glycans. The presence of either

Table 3. *Molar ratios of monosaccharide methyl ethers present in the methanolysates of the permethylated neutral glycopeptides isolated from human milk sIgA*
The molar ratios were calculated on the basis of two residues of 3,4,6-Me_3Man (3,4,6-tri-*O*-methylmannose). IV-1 and IV-3 obtained in trace amounts were not methylated. The available quantities were used for 500-MHz ^{1}H-NMR spectroscopic analysis

Monosaccharide methyl ethers	Molar ratio in asialoglycopeptide fractions								
	I	II	III	IV-2	IV-4	IV-5	V	VI	VII
2,3,4-Me_3Fuc	0.05	0.36	0.10	0.60	0	1.37	1.34	1.59	2.84
2,3,4,6-Me_4Gal	0.21	0.28	0.34	0.80	0.92	0.97	1.64	2.13	1.72
2,4,6-Me_3Gal	0	0	0	0	0	0	0.84	1.79	1.53
3,4,6-Me_3Gal	0	0	0	0	0	0	0.05	0.39	0.26
2,3,4-Me_3Gal	0	0	0	0	0	0	0	0.20	0
2,3,4,6-Me_4Man	0.92	0.30	0	0	0	0	0	0	0
3,4,6-Me_3Man	2	2	2	2	2	2	2	2	2
2,4-Me_2Man	0.78	0.75	0.28	0	0.31	0.15	1.13	1.09	0.97
2-Me_1Man	0.42	0.22	0.70	0.90	0.63	0.70	0.44	0	traces
3,4,6-Me_3GlcNAc(Me)	1.43	2.10	2.62	2.00	1.78	1.89	0.29	0	traces
3,6-Me_2GlcNAc(Me)	1.33	1.26	1.36	1.85	2.15	1.31	2.42	3.28	2.37
6-MeGlcNAc(Me)	0	0	0	0	0	0.37	1.43	0.88	1.63
3-MeGlcNAc(Me)[a]	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
4-MeGlcNAc(Me)	0	0	0	0	0	0	0	0.31	0

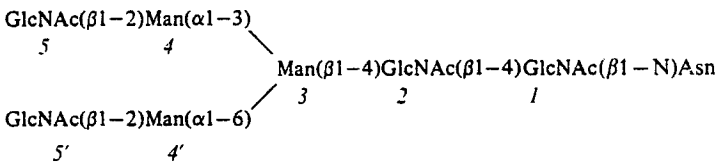
[a] n.d.: not determined; the methylated derivative of this GlcNAc residue could not be quantified after methanolysis [16] due to the fact that under the applied conditions of methanolysis the linkage between GlcNAc-1 and the asparagine residue is not completely cleaved.

3-Me_1GlcNAc(Me) or 6-Me_1GlcNAc(Me) or both derivatives points to the substitution of these GlcNAc residues by Fuc in C6 and C3 position, respectively, besides a substitution at C4.

500-MHz ^{1}H-NMR spectroscopy

To arrive at a more definite evaluation of the heterogeneity that appeared to occur in the GP-N fractions from the data of sugar and methylation analyses (see Tables 1–3), high-resolution ^{1}H-NMR spectroscopy was applied. First, 500-MHz ^{1}H-NMR spectra of the main fractions I–VII were recorded, after dissolution of the material in ^2H_2O, at 27°C. The spectrum of fraction V, as a typical example of the larger-size glycopeptides, is depicted in Fig. 2. This figure gives an impression of how structural heterogeneity manifests itself in an NMR spectrum. Fraction III, the spectrum of which is given in Fig. 3, is representative for the smaller-size glycopeptides. The NMR-spectra of most of the fractions I–VII, like Fig. 2 and 3, showed a number of signals in the structural-reporter-group regions, having non-integral intensity ratios, thereby clearly indicating the samples to be mixtures of two or more components (see also legend to Fig. 2) [19, 20]. After ConA and *L. culinaris* agglutinin chromatography of the major fraction IV, the resulting subfractions, IV-1 to IV-5, were also investigated by 500-MHz ^{1}H-NMR spectroscopy. The spectrum of the most abundant namely IV-4, is depicted in Fig. 4. The spectra of these subfractions showed considerably less complex patterns, indicating that the extent of heterogeneity was less than that of total fraction IV.

Notwithstanding the difficulties that are encountered in the interpretation of an NMR spectrum of a complex mixture of components, primary structures could be deduced for the constituents of all GP-N fractions, including the sites of heterogeneity, by ^{1}H-NMR spectroscopy, utilizing the information available from (Tables 1–3) sugar and methylation analyses. In general, it could be concluded from the spectra of the various GP-N fractions, that their glycopeptide components (except the minor, oligomannoside-type constituents of I and II, see later) have in common the following heptasaccharide:



Several sites (see Table 4) of heterogeneity in extensions of this structure were observed among the various fractions.

Site A. A variable amount of Fuc was present at GlcNAc–1 in (α1–6) linkage in the main fractions (indicated by mole fraction A, in which $0 \leq A \leq 1$).

Site B. The intersecting GlcNAc-9 residue, (β1–4)-linked to Man-3, was present in many but not in all structures forming part of the GP-N main fractions (that is: $0 \leq B \leq 1$). Going from fraction I to VII, the molar amount of GlcNAc-9 gradually decreased, whereas the aforementioned common element was found to be more and more elongated at the GlcNAc-5 and/or GlcNAc-5′ residues.

Site C. Some fractions were found to contain additional Gal-6 and/or Gal-6′ (β1–4)-linked to GlcNAc-5 and GlcNAc-5′, respectively ($0 \leq C \leq 1$).

Site D. Gal being present, an additional Fuc residue was encountered in some of the components, in particular of the larger-size fractions, in (α1–3)-linkage to GlcNAc ($0 \leq D \leq 1$).

Site E. In fractions V to VII, part of the Gal-6 and/or Gal-6′ residues bore an additional *N*-acetyllactosamine unit in (β1–3)-linkage ($0 \leq E \leq 2$), which in turn might be extended (see site F).

Site F. A Fuc residue was present in some cases in (α1–3)-linkage to the GlcNAc residue of this unit ($0 \leq F \leq 1$).

The detailed structural elements listed above, and their designations are summarized in the heading of Table 4.

Employing 500-MHz ^{1}H-NMR spectroscopy, for each of the GP-N (sub)fractions, the sites of heterogeneity could be defined in terms of the mole fractions of the elements at the

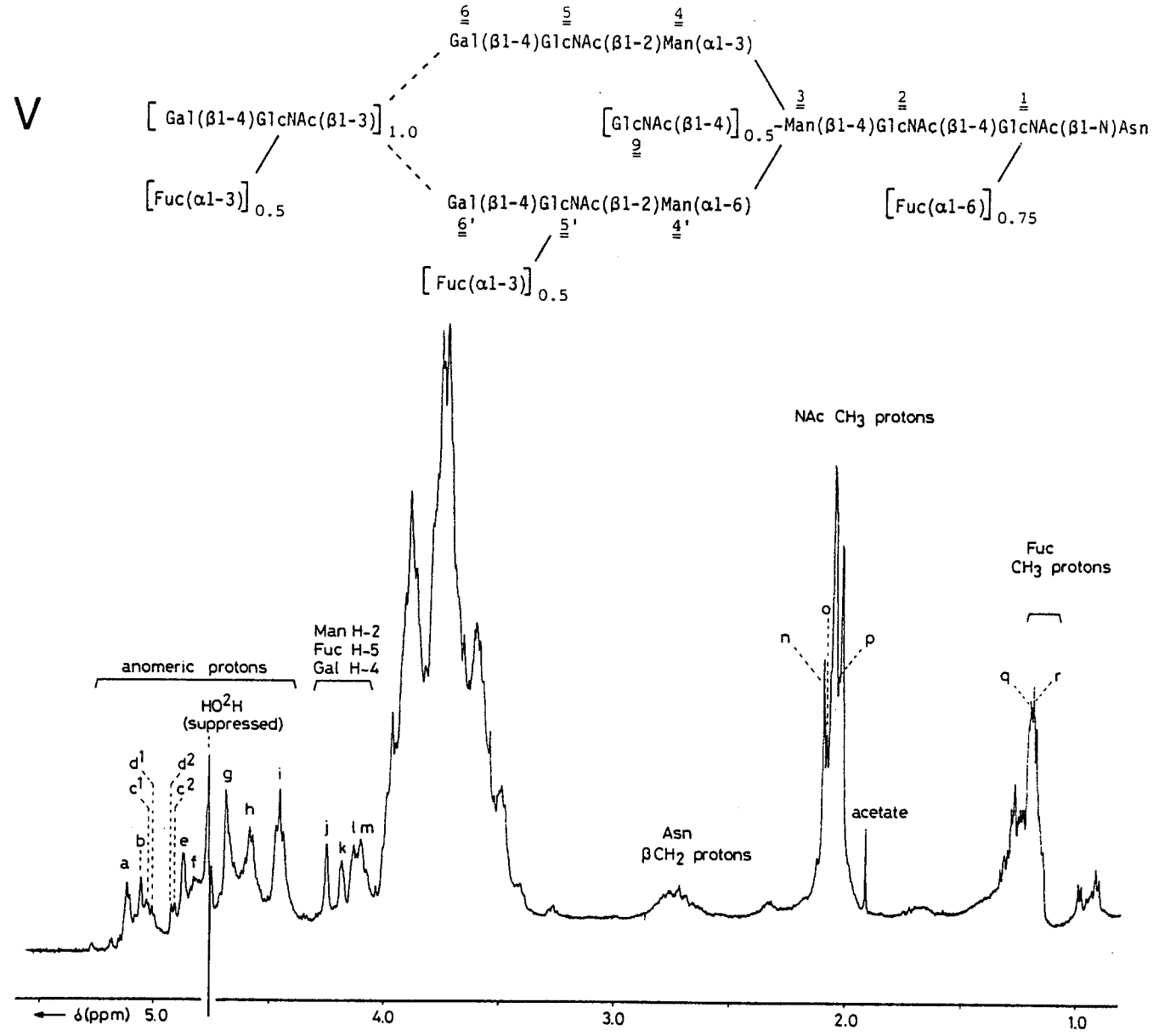


Fig. 2. *500-MHz ^{1}H-NMR spectrum of glycopeptide fraction V obtained from the neutral carbohydrate portion of sIgA, by Bio-Gel P-30 chromatography.* Measuring conditions: ^2H_2O; 27°C; p^{2}H ≈ 7; suppressing of the HO^{2}H signal by a WEFT pulse sequence; spectral width 2600 Hz; 16000 data points. (a) H-1 of Fuc(α1 – 3) and H-1 of Man-4 (the latter only without GlcNAc-9 being present). (b) H-1 of Man-4 (with GlcNAc-9 present) and part of the H-1 doublet of GlcNAc-1. (c^1) H-1 of Man 4′ [with GlcNAc-9 and with Fuc(α1 – 3) linked to GlcNAc-5′]. (d^1) H1 of Man-4′ [with GlcNAc-9 but without Fuc(α1 – 3) linked to GlcNAc-5′]. (c^2) H-1- of Man-4′ [without GlcNAc-9 but with Fuc(α1 – 3) linked to GlcNAc-5′]. (d^2) H-1 of Man-4′ [without GlcNAc-9 and without Fuc(α1 – 3) linked to GlcNAc-5′]. (e) H-1 of Fuc(α1 – 6) and H-1 of Man-4′ (the latter only in absence of GlcNAc-9). (f) H-5 of Fuc(α1 – 3). (g) H-1 of Man-3 (with GlcNAc-9 present); and, at the right wing, H-1 of GlcNAc(β1 – 3) and H-1 of GlcNAc-2 [if Fuc(α1 – 6) at GlcNAc-1]. (h) H-1 of GlcNAc-2 (without Fuc at GlcNAc-1), GlcNAc-5 and GlcNAc-5′. (i) H1 of Gal-6, Gal-6′, the additional Gal(β1 – 4), and of GlcNAc-9. (j) H-2 of Man-3 (without 9) and H-2 of Man-4 (with 9). (k) H-2 of Man-4 (without 9) and H-2 of Man-3 (with 9). (l) H-4 of Gal-6 and/or Gal-6′, bearing an additional *N*-acetyllactosamine in (β1 – 3) linkage. (m) H-2 of Man-4′ and H-5 of Fuc(α1 – 6). (n) NAc of GlcNAc-2 in the presence of Fuc(α1 – 6) at GlcNAc-1. (o) NAc of GlcNAc-2 in the absence of Fuc(α1 – 6) at GlcNAc-1. (p) NAc of GlcNAc(β1 – 3) in the presence of Fuc(α1 – 3) at this residue. (q) CH_3 of Fuc(α1 – 6). (r) CH_3 of Fuc(α1 – 3). The heterogeneity indicated in the overall structure on top of the spectrum, is illustrated by the multiplicity of several signals (for example, site A: n/o; site B: c^1 + d^1/c^2 + d^2) or by typically affected signals (site E: l; site F: p) (compare Table 4)

extreme values of their index. For index = 0, a certain element is considered to be present as a 'fundamental' element; for index = 1, it is denoted as an 'extended' element. Discrimination between the pairs of each set could be achieved on the basis of the characteristic NMR features of sites A to F, which have been compiled in Table 4. They are largely in agreement with those mentioned for similar structural elements [19, 20].

For the determination of the molar ratios of a fundamental and its corresponding extended structural element, mainly the intensity ratio of signals affected in chemical shift by this extension, is very useful. The relative intensities of the reporter groups of the extending residue(s) itself (themselves), might be of help, if separately observable. It should be noted that the effects of all extensions listed in Table 4 on the ^{1}H-NMR

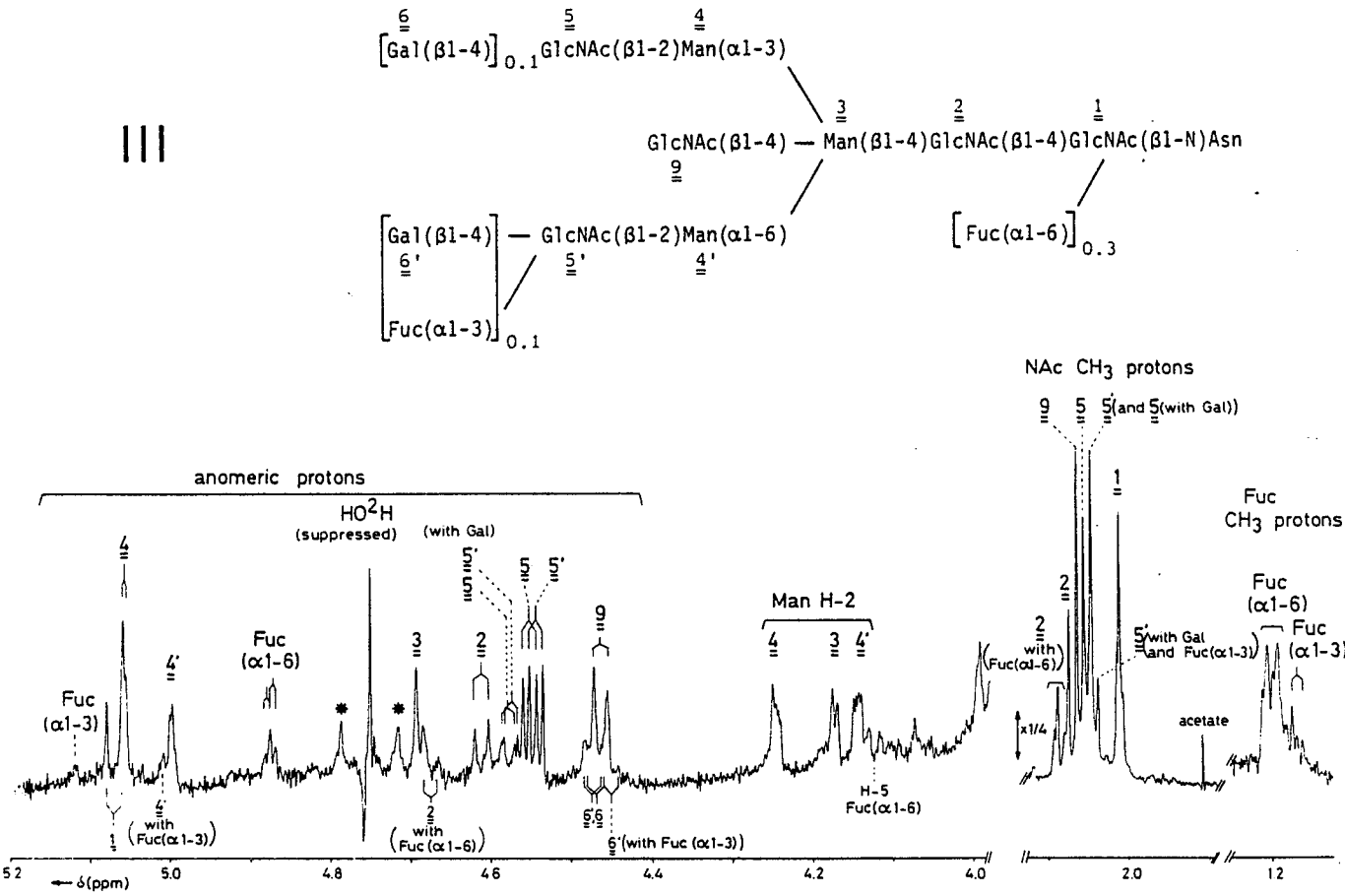


Fig. 3. *Structural-reporter-group regions of the resolution-enhanced 500-MHz ^{1}H-NMR spectrum of glycopeptide fraction III, obtained from the neutral carbohydrate portion of sIgA by Bio-Gel P-30 chromatography.* Measuring conditions: see legend to Fig. 2. The numbers in the spectrum refer to the corresponding residues in the structure. The relative-intensity scale of the *N*-acetyl proton region deviates from that of the other parts of the spectrum. Asterisks indicate spinning side bands of the HO^{2}H signal

parameters of structural-reporter groups of neighbouring residues are independent from each other. This implies that it can only be deduced from the ^{1}H-NMR spectrum of a given fraction, to which extent each of the elongations occurs, but not whether they occur together in one compound. Furthermore, it is worth mentioning that for reliable spectral integration only the nondisturbed spectra can be used (that is not the WEFT spectra [21] and, most importantly, not the spectra resolution-enhanced by computer techniques). A sufficient resolution in conjunction with comparable line-width (resulting in similar distortions by the WEFT technique and the Lorentzian-to-Gaussian transformation) made the following reporter groups most suited for determination of rough ($\pm 10\,\%$) centesimal proportions.

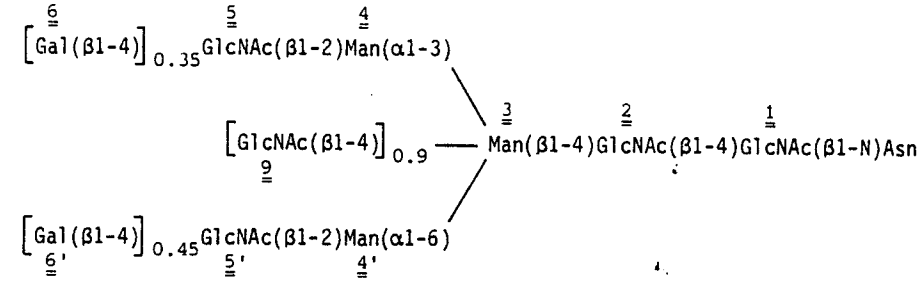
Site A. The amount of Fuc (α1 − 6)-linked to GlcNAc-1 relative to the unsubstituted GlcNAc-1 could be deduced unambiguously from the intensity ratio of the *N*-acetyl signals of GlcNAc-2 at $\delta = 2.093$ ppm and $\delta = 2.080$ ppm, respectively. In most spectra, also the ^{1}H-1 signals of GlcNAc-2 at $\delta = 4.68$ ppm and $\delta = 4.61$ ppm were undisturbed. This is illustrated by signals n and o in Fig. 2, and also in Fig. 3.

Site B. The presence of GlcNAc-9 is expressed in a vast number of well established [20] shift effects, in particular on the H-1 and H-2 signals of the mannose residues 3, 4 and 4′. The ratio of the signals at $\delta = 5.12$ ppm to $\delta = 5.05$ ppm (that is, H-1 of Man-4) as well as that of the signals at $\delta = 4.92$ ppm to $\delta = 5.00$ ppm (H-1 of Man-4′) appeared to be useful for estimation of the relative amount of GlcNAc-9 present in a mixture [see Fig. 2, signals $(c_1 + d_1)$ as compared to $(c_2 + d_2)$; and Fig. 4].

Site C. In structures that end in GlcNAc-5 and GlcNAc-5′, the H-1 signals of these residues were observed separately (see for example Fig. 4), at $\delta = 4.552$ ppm and $\delta = 4.543$ ppm, respectively. Also, the *N*-acetyl protons resonated clearly apart from each other, at $\delta = 2.058$ ppm and $\delta = 2.048$ ppm, respectively. The assignment of these H-1 and *N*-acetyl signals to either GlcNAc-5 or GlcNAc-5′ was based upon comparison with the spectral data of a hexasaccharide and a heptasaccharide isolated from the urine of a patient with Sandhoff's disease [20, 22], having structures similar to the aforementioned common element of GP-N glycopeptides and its extension with GlcNAc-9, but missing the GlcNAc(β1-N)Asn moiety. In the spectra of the reducing oligosaccharides, only the high-field signals within each set were doubled in the anomeric intensity ratio ($\alpha:\beta = 2:1$) and were therefore attributed to GlcNAc-5′ [20, 22].

This provided us with two useful criteria to determine the location of an extending Gal residue in a certain branch, in addition to the difference in chemical shifts between the H-1 signals of Gal-6 and Gal-6′ themselves (see Table 4): the chemical shifts of the couple of H-1 and *N*-acetyl signals which were not affected by the presence of a Gal residue, were decisive for the terminal GlcNAc residue to be 5 or 5′. Recognition of the reporter-group signals of these terminal residues was

IV-4



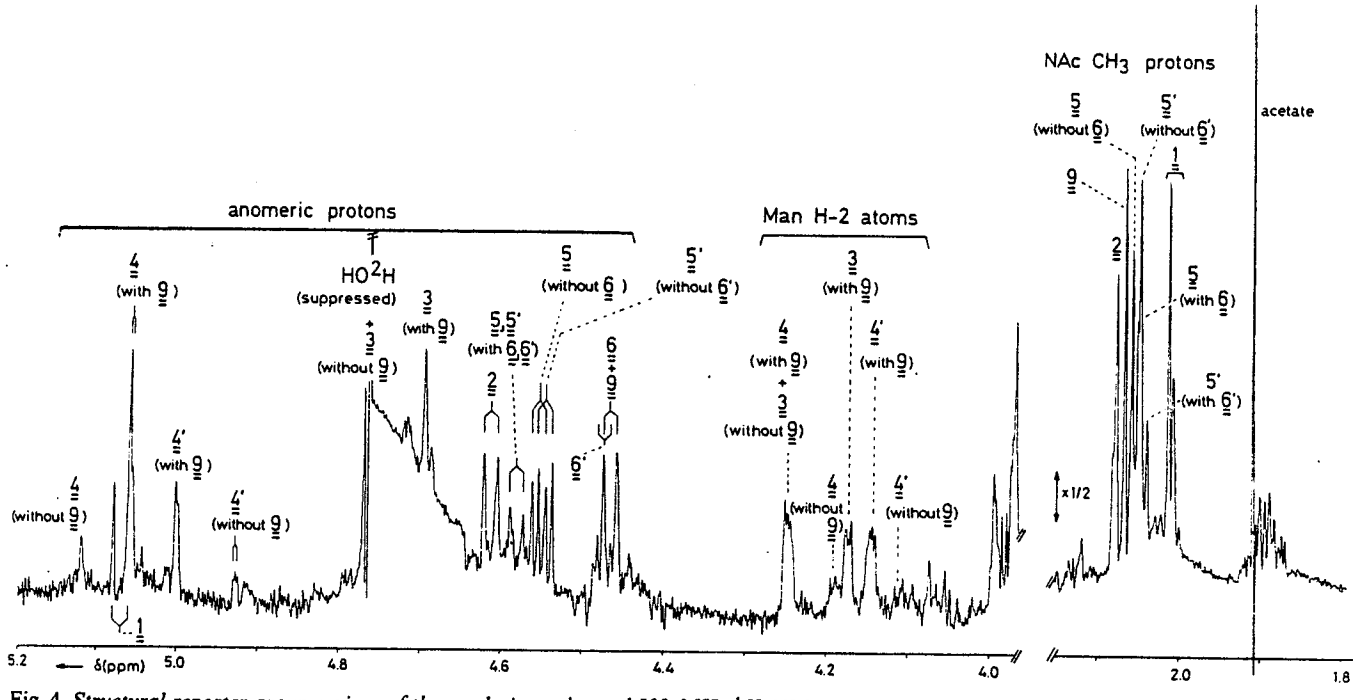


Fig. 4. *Structural-reporter-group regions of the resolution-enhanced 500-MHz ^{1}H-NMR spectrum of glycopeptide subfraction IV-4, obtained from sIgA GP-N fraction IV by lectin affinity chromatography (see Fig. 1).* The spectrum was recorded under the conditions described in the legend to Fig. 2. The numbers in the spectrum refer to the corresponding residues in the structure. The relative-intensity scale of the *N*-acetyl proton region deviates from that of the other part of the spectrum

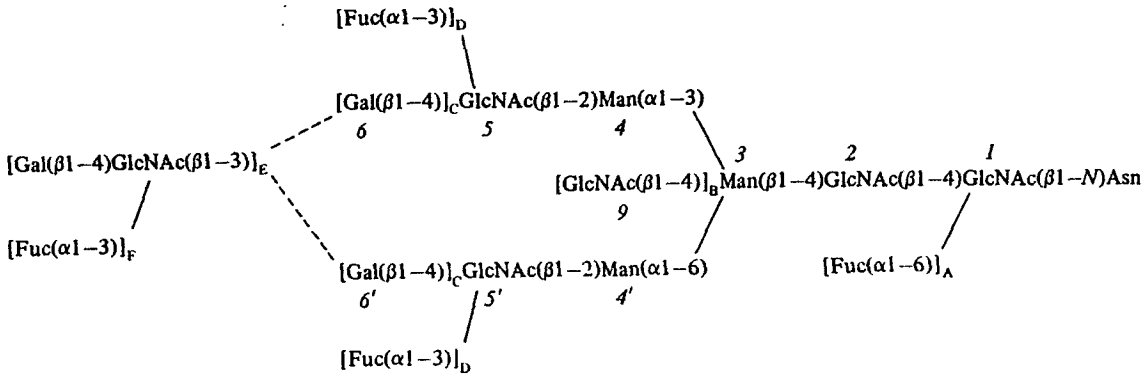
facilitated by their relatively sharp lines (see Fig. 3 and 4). It may be mentioned that, in the case that both branches are galactosylated, the H-1 atoms of GlcNAc-5 and GlcNAc-5′ became equivalent, while the difference in chemical shift of their *N*-acetyl signals remained.

The amount of localizable Gal present could be estimated in all spectra most readily from the ratio of the signals at $\delta = 4.54$ ppm and $\delta = 4.58$ ppm. Fig. 3 gives an illustration of the difference in linewidth of both signals, induced by the attachment of Gal; this difference in linewidth makes accurate integration of the spectrum impossible after computer resolution enhancement, because signals with different linewidths are treated differently in this procedure.

Site D. The effects of the attachment of a Fuc residue in (α1−3)-linkage to GlcNAc of a peripheral *N*-acetyllactosamine unit (see Table 4) were in accord with those described previously [19, 20, 25, 26]. Most indicative of the relative amount of this type of Fuc present at a certain GlcNAc residue, was the intensity ratio of the *N*-acetyl signals of the GlcNAc with and without attached Fuc, respectively. These signals were sufficiently separated ($|\Delta\delta| \approx 0.01$ ppm [25]) to be observed apart from each other, even without resolution enhancement. Independently, the intensity ratio of the α-Man H-1 signal affected by fucosylation of the neighbouring GlcNAc, and the corresponding α-Man H-1 signal of the unsubstituted branch ($|\Delta\delta| \approx 0.012$ ppm) (cf. [27]), was, in some of the spectra, a useful means of quantifying the amount of (α1−3)-linked Fuc (see, for example, Fig. 2 and 3).

Site E. The larger-size glycopeptides comprising fractions V to VII contained one or more additional *N*-acetyllactosamine units in (β1−3)-linkage to Gal-6 and/or Gal-6′. The latter are characterized by a GlcNAc H-1 signal at $\delta \approx 4.70$ ppm, an *N*-acetyl signal at $\delta \approx 2.040$ ppm and, most significantly, by the H-4 signal of the substituted Gal residue, emerged from the bulk of skeleton protons ($\delta \approx 3.92$ ppm) to $\delta = 4.15$ ppm. These chemical shifts and shift effects are in complete agreement with those described [20] for a similar extension of an *N*-acetyllactosamine unit, observed for glyco-asparagines isolated from the urine of patients with aspartyl-glucosaminuria (unpublished results of J. C. Michalski, J. Montreuil, G. Strecker, H. van Halbeek, L. Dorland, and J. F. G. Vliegenthart cited in [20]). Unfortunately, none of the above-mentioned signals provided a suitable criterion for localization of an additional *N*-acetyllactosamine unit on a certain branch. Moreover, quantification of the number of units present was not easy since in many spectra, the aforementioned signals

Table 4. *^{1}H-NMR characteristics of the structural elements of the neutral carbohydrate moiety of human milk sIgA; heterogeneity has been found to occur at sites A to F*



Chemical shifts are mean values given in ppm downfield from sodium 4,4-dimethyl-4-silapentane-1-sulphonate for neutral, ^2H_2O solutions at 27 °C; all data were acquired at 500 MHz. Accuracy = 0.003 ppm. Standard deviation = 0.005 ppm

Site	Fundamental element	^{1}H Chemical shift	Reporter group		^{1}H Chemical shift	Extended element
		ppm			ppm	
A	.–4)GlcNAc(β1–4)GlcNAc(β1–*N*)Asn 2 1	5.07[a] 2.01[a] 4.610 2.080 – – –	GlcNAc-1 GlcNAc-1 GlcNAc-2 GlcNAc-2 Fuc(α1–6) Fuc(α1–6) Fuc(α1–6)	H-1 NAc H-1 NAc H-1 H-5 CH_3	5.07[a] 2.01[a] 4.68 2.093 4.87[a] 4.12 1.20[a]	2 1 .–4)GlcNAc(β1–4)GlcNAc(β1–*N*)Asn Fuc(α1–6)
B	4 .–2)Man(α1–3) Man(β1–4)GlcNAc. . . .–2)Man(α1–6) 3 2 4′	5.12 4.19 4.77 4.250 – – – 4.920 4.11	Man-4 Man-4 Man-3 Man-3 GlcNAc-9 GlcNAc-9 'triplet'[b] Man-4′ Man-4′	H-1 H-2 H-1 H-2 H-1 NAc H-1 H-2	5.05 4.245 4.695 4.175 4.465 2.066 3.27 5.00 4.145	4 .–2)Man(α1–3) GlcNAc(β1–4)–Man(β1–4)GlcNAc. . . 9 3 2 .–2)Man(α1–6) 4′
C	GlcNAc(β1–2)Man(α1–3) 5 4 Man(β1–4). . . GlcNAc(β1–2)Man(α1–6) 3 5′ 4′	4.552 2.058 – 4.543 2.049 –	GlcNAc-5 GlcNAc-5 Gal-6 GlcNAc-5′ GlcNAc-5′ Gal-6′	H-1 NAc H-1 H-1 NAc H-1	4.582 2.052 4.467 4.582 2.046 4.472	Gal(β1–4)GlcNAc(β1–2)Man(α1–3) 6 5 4 Man(β1–4). . 3 Gal(β1–4)GlcNAc(β1–2)Man(α1–6) 6′ 5′ 4′

D	Gal(β1–4)GlcNAc(β1–2)Man(α1–3) 6 5 4 Man(β1–4)... 3 Gal(β1–4)GlcNAc(β1–2)Man(α1–6) 6′ 5′ 4′	5.12 2.052 4.467 – – – 4.922 2.046 4.472	Man-4 GlcNAc-5 Gal-6 Fuc(α1–3) Fuc(α1–3) Fuc(α1–3) Man-4′ GlcNAc-5′ Gal-6′	H-1[c] NAc H-1 H-1 H-5 CH_3 H-1[c] NAc H-1	5.11 2.044 4.445 5.11 4.83 1.17 4.908 2.038 4.445	Fuc(α1–3) Gal(β1–4)GlcNAc(β1–2)Man(α1–3) 6 5 4 Man(β1–4)... 6′ 5′ 4′ 3 Gal(β1–4)GlcNAc(β1–2)Man(α1–6) Fuc(α1–3)
E	Gal(β1–4)GlcNAc(β1–2)Man(α1–. 6/6′ 5/5′ 4/4′	4.47 3.92 – – –	Gal-6(′) Gal-6(′) GlcNAc(β1–3) GlcNAc(β1–3) Gal(β1–4)	H-1 H-4 H-1 NAc H-1	4.48[d] 4.15[d] 4.72[d] 2.039 4.47[e]	Gal(β1–4)GlcNAc(β1–3) Gal(β1–4)GlcNAc(β1–2)... 6/6′ 5/5′
F	Gal(β1–4)GlcNAc(β1–3)Gal(β1–4)... 6/6′	4.48[d] 2.039 4.47[d] – – –	Gal-6(′) GlcNAc(β1–3) Gal(β1–4) Fuc(α1–3) Fuc(α1–3) Fuc(α1–3)	H-1 NAc H-1 H-1 H-5 CH_3	4.47[d] 2.028 4.445 5.11 4.83 1.17	Gal(β1–4)GlcNAc(β1–3)Gal(β1–4)... 6/6′ Fuc(α1–3)

[a] Precise value depends mainly on the composition of the peptide moiety; heterogeneity of the latter is reflected in the multiplicity of this signal.

[b] The emergence of a triplet-like signal at $\delta = 3.27$ ppm out of the bulk signal of sugar skeleton is known to be associated with the occurrence of the intersecting GlcNAc-9. So far, this 'triplet' has not been assigned to a particular proton, yet [23, 24].

[c] In case of an intersected biantennary structure, H-1 of Man-4 shifts upfield from $\delta = 5.05$ ppm to $\delta = 5.03$ ppm, whereas surprisingly, H-1 of Man-4′ shifts downfield from $\delta = 5.00$ ppm to $\delta = 5.02$ ppm, upon fucosylation of GlcNAc-5 and -5′, respectively.

[d] Chemical shift values for reporter groups of additional *N*-acetyllactosamine units, and for those affected by their attachment, could not be determined more accurately (± 0.015 ppm), due to the complexity of the spectra of fractions V, VI, VII (see Fig. 2).

Table 5. *Relative abundance of fundamental and extended structural elements at heterogeneity sites A to F in the neutral glycopeptide fractions derived from human milk sIgA*

Relative abundances are based on NMR spectral integration (for signals that are decisive for each particular element, see text). Estimated accuracy is about 10%. For designation of structural elements, see Table 4 (heading). In the glycopeptides listed under E + F, no additional *N*-acetyllactosamine units without Fuc linked to GlcNAc were found; therefore, they are considered together in this column. Fraction I consisted of 50% oligomannoside-type glycopeptides, and 50% of the *N*-acetyllactosamine-type glycopeptides; only the latter are considered in this table. This ratio of 50:50 is based on the intensity of the Man-3 H-2 signals for both types of compound at $\delta = 4.23$ ppm and $\delta = 4.17$ ppm, respectively. In column D, the percentage of extension of the Gal(β1 – 4)GlcNAc units mentioned at column C is given; therefore, if only 15% of GlcNAc-5′ is bearing a Gal but at the same time a Fuc residue, column D contains 100% proportion of extension. Fraction VI and VII were found to be contaminated with structures containing the Le^b-blood group determinant, that is a type-I structure, namely Fucα1 – 2Galβ1 – 3(Fucα1 – 4)-GlcNAcβ1-· (see text). n.d., not determined: exact numbers could not be determined, due to the presence of a frequently occurring nonprotein, noncarbohydrate contaminant giving rise to i.a. a quartet at $4.08 < \delta < 4.12$ ppm

Glycopeptide (sub)fraction	Relative abundance of element at site													
	A		B		C				D				E + F	
					upper branch		lower branch		upper branch		lower branch			
	fund./ext.		fund./ext.		fund./ext.		fund./ext.		fund./ext.		fund./ext.		fund./ext.	
	%													
I	80	20	–	100	100	–	100	–	–	–	–	–	–	–
II	70	30	–	100	100	–	85	15	–	–	–	100	–	–
III	70	30	–	100	100	–	80	20	–	–	50	50	–	–
IV	40	60	10	90	80	20	70	30	100	–	–	100	–	–
IV-1	100	–	–	100	–	100	100	–	100	–	–	–	–	–
IV-2	–	100	–	100	–	100	100	–	100	–	–	–	–	–
IV-3	100	–	–	100	–	100	100	–	100	–	–	–	–	–
IV-4	100	–	10	90	65	35	55	45	100	–	100	–	–	–
IV-5	–	100	–	100	75	25	65	35	100	–	10	90	–	–
V	25	75	50	50	–	100	–	100	95	5	50	50	50	50
VI	30	70	95	5	–	100	–	100	90	10	50	50	n.d.	n.d.
VII	30	70	95	5	–	100	–	100	100	–	50	50	–	100

could not be observed free from other resonances (see Fig. 2). The H-4 signal of Gal at $\delta = 4.15$ ppm is in most cases of some use for this purpose.

Site F. The NMR features of the attachment of Fuc in (α1 – 3)-linkage to GlcNAc forming part of such an additional *N*-acetyllactosamine unit, could be treated in the same way as described above for site D (see Table 4). The upfield shift, introduced by the Fuc attachment, upon the *N*-acetyl signal of the (β1 – 3)-linked GlcNAc ($\Delta\delta \approx -0.01$ ppm) results in a rather highfield position of the latter ($\delta \approx 2.028$ ppm) which makes this signal clearly observable in most cases (see Fig. 2, signal p).

Using the decisive features mentioned above, the abundances of extended elements relative to their fundamental counterparts could be derived from the 500-MHz ^{1}H-NMR spectra of the various neutral glycopeptide fractions obtained from sIgA. However, it should be emphasized again that, since more than one type of heterogeneity was concerned in almost any spectrum, the procedure of arriving at the molar ratios was not as straightforward as might appear from the 'rules' A – F, due to overlap of resonances necessary for integration (see Fig. 2.)

The estimated compositions of the glycopeptide preparations, based on integration of the appropriate parts of the NMR spectra, have been compiled in Table 5.

The overall structures, resulting from a combination of the results of sugar analysis, methylation/mass spectrometry and NMR spectroscopy are listed in Fig. 5 and 6.

The following features of some fractions deserve special mention since they could not be incorporated in Fig. 5 and 6, nor in Tables 4 and 5. In addition to the structure for fraction I included in Fig. 5, about 40 – 50% of this fraction consisted of oligomannoside type glycopeptides varying in chain length from $(Man)_5(GlcNAc)_2Asn$ to $(Man)_7(GlcNAc)_2Asn$. This was evident from the presence of Man H-1 signals in the NMR spectrum at positions characteristics for this type of structure (e.g. at $\delta = 5.402$ ppm, for H-1 of Man-A bearing Man-D_2; at $\delta = 5.346$ ppm, for Man-4 bearing Man-C and at $\delta = 5.335$ ppm for Man-4 bearing the C-D_1 disaccharide unit; at $\delta = 5.303$ ppm for Man-C, substituted by Man-D_1 and $\delta = 5.142$ ppm for Man-B, bearing Man-D_3) [20]. This observation was in line with the results from methylation analysis, showing the presence of permethylated (2,3,4,6-Me_4Man) mannose (Table 3) in glycopeptide fraction I. The latter pointed to the occurrence of terminal Man residues. Treatment of this fraction with endo-*N*-acetyl-β-D-glucosaminidase D followed by thin-layer chromatography showed that about 40% of the material was hydrolyzed. The fractionation of glycopeptide I on a ConA-Sepharose column (under the same conditions as described above) also confirmed the NMR results. Three peaks were obtained. The first was eluted with the starting buffer and contained Fuc, Gal, Man and GlcNAc in the molar ratios 0.3:0.5:3:5 respectively. The amount of the second peak eluted with 0.01 M methyl α-D-glucopyranoside was too small to allow sugar analysis. The third peak eluted with 0.3 M methyl α-D-glucopyranoside contained 6 – 8 Man for 2 GlcNAc residues.

As to glycopeptide fraction IV-5, it may be mentioned that the absence of a 4,6-Me_2GlcNAc derivative in the methylation analysis (Table 3) led independently to the conclusion that glycopeptide IV-5 possessed a second Fuc residue in the same branch as where Gal was located. The presence of one 2,4,6-

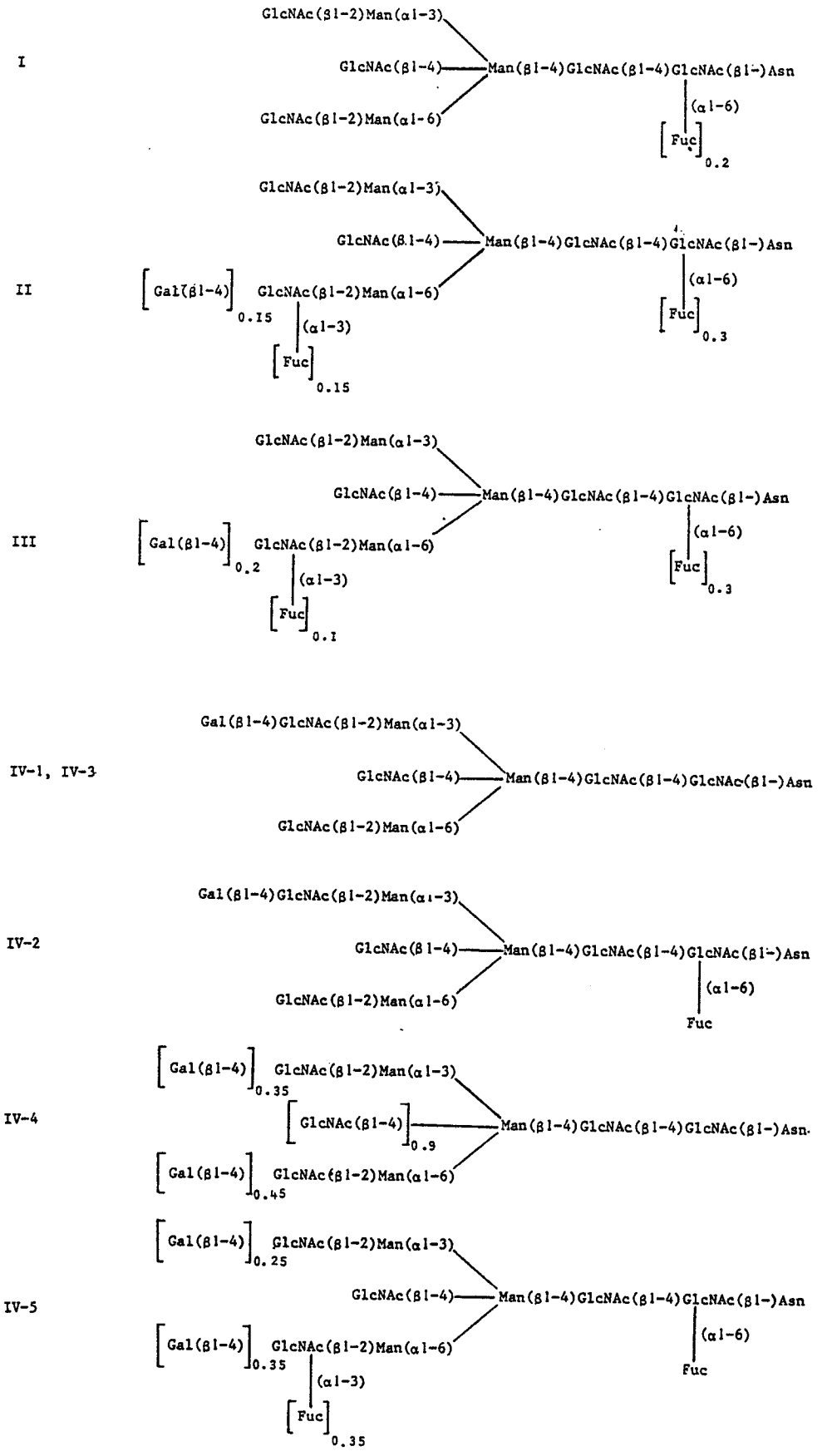


Fig. 5. *Structures of the* N-*acetyllactosamine-type asialoglycopeptides from human milk sIgA*. Values associated to brackets refer to the relative abundance of the structural element (see Table 5)

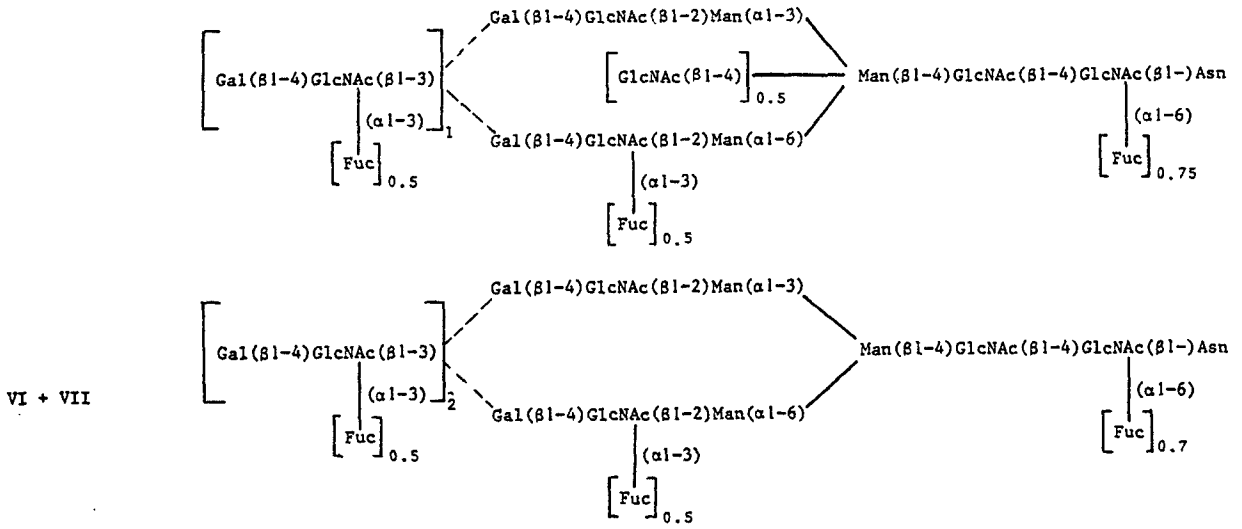


Fig. 6. *Structures of the extended biantennary* N-*acetyllactosamine-type asialoglycopeptides from human milk sIgA.* Values associated to brackets refer to the relative abundance of the structural element (see Table 5)

Me_3Gal residue after permethylation of glycopeptide V and of two for glycopeptides VI and VII (see Table 3) demonstrated that 1 and 2 supplementary units of *N*-acetyllactosamine respectively would be substituting the Gal residues 6 and/or 6′ at the C-3 position. This is supported by the results of quantification by NMR (Table 5).

Mass spectrometry led to the detection of a small amount of 3,4,6-Me_3Gal in the methylation analysis of glycopeptides VI and VII showing that structures containing a C-2-substituted Gal residue were present in each of the mixtures in relative amounts of $\approx 10\%$. The 500-MHz ^{1}H-NMR spectra of fractions VI and VII revealed low-intensity H-1 signals at $\delta = 5.189$ ppm and at $\delta = 5.151$ ppm, which are known to be characteristic for Fuc in the structural moiety Fucα1 – 2Gal β1 – 3GlcNAcβ1 – 3 without and with an additional Fuc (α1 – 4)-linked to GlcNAc, respectively [the Fuc(α1 – 4) was characterized by its H-1 signal at $\delta = 5.032$ ppm]. The corresponding Fuc(α1 – 2) H-5 signal was found at $\delta = 4.29$ ppm, whereas Fuc CH_3 doublets were observed at $\delta = 1.28$, 1.26 and 1.24 ppm. These values showed close resemblance to those observed for milk oligosaccharides containing the blood group H (type I), and the Lewis^b blood-group determinants, respectively (unpublished results). Fraction VI indeed contained Lewis^b activity. Haemagglutination up to a dilution of 1 in 4 was obtained with about 80 µg of fraction VI confirming the presence of a Fucα1 – 2Galβ1 – 3(Fucα1 – 4)GlcNAc-R component.

DISCUSSION

Whilst being less abundant than the sialoglycopeptides in human milk sIgA, the asialoglycopeptide fraction is far more heterogeneous. This heterogeneity was also noted for the *O*-glycosidic fraction [1, 2] and may be due to the fact that milk from different donors was pooled and to an intrinsic property of the sIgA. The heterogeneity of the asialoglycopeptides could be fairly well characterized in terms of primary structures by applying a combination of sugar analysis, high-resolution ^{1}H-NMR spectroscopy, and permethylation analysis on glycopeptide fractions separated according to their size and, partly, according to their binding affinity for some lectins (see Fig. 5 and 6).

Regarding the applied fractionation procedures, it may be concluded that it was possible to separate structures differing by two or more monosaccharide units adequately by Bio-Gel P-30 gel filtration. However, structures that differed by just one additional monosaccharide, and structural isomers, could not be resolved. The *Lens culinaris* lectin again proved [7, 8, 28] to be extremely specific for Fuc (α1 – 6)-linked to GlcNAc-1, as can be concluding from Tables 2 and 5 (column A). The separation on ConA was less simple. For instance, fractions IV-1 and IV-3 were separated on ConA but had identical glycan structures (Fig. 5). The separation was probably due to differences in their attached peptide chain since hydrophobic amino acid residues have been shown to interfere with ConA fractionation [29]. It is also interesting to note that not only GlcNAc-9 but also the presence of Gal-6 or Gal-6′ apparently influences the ConA binding affinity of the glycopeptides, giving rise to separation on the basis of the location of the Gal residue (see Fig. 5).

Most of the fractions analyzed were heterogeneous as was apparent from non-integral ratios in sugar analysis, methylation analysis, as well as intensities of reporter-group-signals in the NMR spectra. High-resolution ^{1}H-NMR spectroscopy has proved to be capable of elucidating primary structures even in complex mixtures. Besides the usual parameters, namely chemical shifts, coupling constants and linewidths, defining position and pattern of structural-reporter group signals, their relative-intensity appeared to be invaluable for mixture analysis.

Oligomannoside-type structures that are present in fraction I as shown by NMR spectroscopy, methylation analysis, the action of endo-*N*-acetyl-β-D-glucosaminidase and ConA-Sepharose chromatography, are ascribed to the contamination of the sIgA preparation by a small amount of sIgM (6 mg of sIgM for 1 g of sIgA). IgM contains 11% carbohydrate and about 40% of its glycan structures are of the oligomannosidic type [30].

In glycopeptide fractions VI and VII a more complex contaminating structure was found (about 20% of fraction VI) possessing a number of additional fucose residues, substituting a galactose residue at C-2 and an *N*-acetylglucosamine at C-4. The structure Fucα1 – 2Galβ1 – 3 (Fucα1 – 4)GlcNAcβ1 – 3-Gal-R is part of one of the free milk oligosaccharides [31, 32]

and should in this case be terminated by a glucose residue. However, the presence of glucose was never demonstrated, whether by methanolysis, methylation after reduction of fraction VI or by NMR. The labelling of this fraction and its subsequent chromatography on lectins having different specificities for fucose may give an answer as to the origin of this molecule. It is either an integral part of sIgA or a contaminant. In the latter case it would have to be considered that it is adsorbed only onto the neutral glycopeptide fraction. Sialylated free oligosaccharides exist in human milk but were not detected contaminating the acidic glycopeptides of sIgA [6].

Both biantennary *N*-acetyllactosamine-type and extended biantennary *N*-acetyllactosamine type structures extended by additional fucosyl *N*-acetyllactosamine residues were present in the GP-N fraction. Most of the former possessed an intersecting *N*-acetylglucosamine residue whereas it is striking that the extended-type structures were practically devoid of this additional *N*-acetylglucosamine. The intersecting *N*-acetylglucosamine residue does not figure in the free secretory piece [5] but is present on the α_1 chains of serum IgA [3] and thus would seem to be specific for the α chains. In addition, we have demonstrated that the neutral glycopeptide fraction is not derived from the acidic fraction by the loss of sialic acid during the purification procedure as no sialoglycopeptides were found possessing the intersecting *N*-acetylglucosamine residue [6].

The extended biantennary *N*-acetyllactosamine type structures described here were not found in the sialoglycopeptide fraction of human milk sIgA [6], nor in the *N*-glycosidic glycan fractions of serum IgA [3] but were, on the contrary, demonstrated in the glycan fraction of the free secretory piece isolated from human milk [5]. This suggests that they are probably only present on the secretory piece bound to the IgA dimers in human milk.

This investigation was supported in part by the *Centre National de la Recherche Scientifique* (C.N.R.S.) (Laboratoire Associé no 217. *Relations structure-fonction des constituants membranaires*), by the *Institut National de la Santé et de la Recherche Médicale* (I.N.S.E.R.M.) (Contracts 134.012 and 832.029) by the Netherlands Foundation for Chemical Research (SON) with financial aid from the Netherlands Organization for the Advancement of Pure Research (ZWO) and by Grant UUKC-OC 79-13 from the Netherlands Foundation for Cancer Research (KWF). A.P. received a grant from the *Fondation Nationale pour la Recherche Médicale*. The authors are obliged to Mme L. Mannessiez and the *Centre Régional de Transfusion Sanguine de Lille* (C.R.T.S.) for blood group activity analysis, and to B. Fournet and J. Paz Parente for their advice given for the micromethylation analysis. They wish to thank O. Kol for the endo-β-*N*-acetylglucosaminidase hydrolysis. The authors gratefully acknowledge the expert technical assistance of Y. Leroy (C.N.R.S. technician), G. Ricart with mass spectrometry and J. P. Decottignies with the sIgA preparation.

REFERENCES

1. Pierce-Crétel, A., Pamblanco, M., Strecker, G., Montreuil, J. & Spik, G. (1981) *Eur. J. Biochem. 114*, 169–178.
2. Crétel, A., Pamblanco, M., Egge, H., Strecker, G., Montreuil, J. & Spik, G. (1979) in *Proc. 5th Int. Symp. Glycoconjugates Kiel* (Schauer, R., Boer, P., Buddecke, E., Kramer, M. F., Vliegenthart, J. F. G. & Wiegandt, H., eds) pp. 26–27, Georg Thieme, Stuttgart.
3. Baenziger, J. & Kornfeld, S. (1974) *J. Biol. Chem. 249*, 7270–7281.
4. Baenziger, J. (1979) *J. Biol. Chem. 254*, 4063–4071.
5. Mizoguchi, A., Mizuochi, T. & Kobata, A. (1982) *J. Biol. Chem. 257*, 9612–9621.
6. Pierce-Crétel, A., Pamblanco, M., Strecker, G., Montreuil, J. & Spik, G., Dorland, L., van Halbeek, H. & Vliegenthart, J. F. G. (1982) *Eur. J. Biochem. 125*, 383–388.
7. Debray, H. & Montreuil, J. (1981) in *Lectins-Biology, Biochemistry, Clinical Biochemistry* (T. C. Bøg-Hansen, ed.) vol. 1, pp 221–230.
8. Debray, H., Decout, D., Strecker, G., Spik, G. & Montreuil, J. (1981) *Eur. J. Biochem. 117*, 41–55.
9. Toyoshima, S., Osawa, T. & Tonomura, A. (1970) *Biochim. Biophys. Acta, 221*, 514–521.
10. March, S. C., Parikh, I. & Cuatrecasas, P. (1974) *Anal. Biochem. 60*, 149–152.
11. Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A. & Smith, F. (1956) *Anal. Biochem. 28*, 350–356.
12. Koide, N. & Muramatsu, T. (1975) *Biochem. Biophys. Res. Commun. 66*, 411–416.
13. Zanetta, J. P., Breckenridge, W. C. & Vincendon, G. (1972) *J. Chromatogr. 69*, 291–304.
14. Finne, J., Krusius, T. & Rauvala, H. (1980) *Carbohydr. Research 80*, 336–339.
15. Paz Parente, J., Leroy, Y., Montreuil, J. & Fournet, B. (1984) *Anal. Biochem.* in the press.
16. Fournet, B., Strecker, G., Leroy, Y. & Montreuil, J. (1981) *Anal. Biochem. 116*, 489–502.
17. Tai, T., Yamashita, K., Ogata-Arakawa, M., Koide, N., Muramatsu, T., Iwashita, S., Inoue, Y. & Kobata, A. (1975) *J. Biol. Chem. 250*, 8569–8575.
18. Bouquelet, S., Strecker, G., Montreuil, J. & Spik, G. (1980) *Biochimie (Paris) 62*, 43–49.
19. Vliegenthart, J. F. G., Van Halbeek, H. & Dorland, L. (1981) *Pure Appl. Chem. 53*, 45–77.
20. Vliegenthart, J. F. G., Dorland, L. & Van Halbeek, H. (1983) *Adv. Carbohydr. Chem. Biochem. 41*, 209–374.
21. Van Halbeek, H., Vliegenthart, J. F. G., Winterwerp, H., Blanken, W. M. & Van den Eijnden, D. H. (1983) *Biochem. Biophys. Res. Commun. 110*, 124–131.
22. Strecker, G., Herland-Peers, M. C., Fournet, B., Montreuil, J., Dorland, L., Haverkamp, J. & Vliegenthart, J. F. G. (1977) *Eur. J. Biochem. 81*, 165–171.
23. Carver, J. P., Grey, A. A., Winnik, F. M., Hakimi, J., Ceccarini, C. & Atkinson, P. H. (1981) *Biochemistry, 20*, 6600–6606.
24. Bruch, R. C. & White III, H. B. (1982) *Biochemistry, 21*, 5334–5341.
25. Van Halbeek, H., Dorland, L., Vliegenthart, J. F. G., Montreuil, J., Fournet, B. & Schmid, K. (1981) *J. Biol. Chem. 256*, 5588–5590.
26. Endo, M., Suzuki, K., Schmid, K., Fournet, B., Karamanos, Y., Montreuil, J., Dorland, L., Van Halbeek, H. & Vliegenthart, J. F. G. (1982) *J. Biol. Chem. 257*, 8755–8760.
27. Spik, G., Strecker, G., Fournet, B., Bouquelet, S., Montreuil, J., Dorland, L., Van Halbeek, H. & Vliegenthart, J. F. G. (1982) *Eur. J. Biochem. 121*, 413–419.
28. Kornfeld, K., Reitman, M. L. & Kornfeld, R. (1981) *J. Biol. Chem. 256*, 6633–6640.
29. Loontiens, F. G., Van Wauwe, J. P., De Gussem, R. & De Bruyne, C. K. (1973) *Carbohydr. Res. 30*, 51–62.
30. Chapman, A. & Kornfeld, R. (1979) *J. Biol. Chem. 254*, 816–823.
31. Kobata, A., Yamashita, K. & Tachibana, Y. (1978) *Methods Enzymol. 50*, 216–220.
32. Smith, D. F., Zopf, D. A. & Ginsburg, V. (1978) *Methods Enzymol. 50*, 221–226.

A. Pierce-Crétel, H. Debray, J. Montreuil, and G. Spik,
Laboratoire de Chimie Biologique (et Laboratoire associé au Centre National de la Recherche Scientifique no 217),
Université de Sciences et Techniques de Lille I, F-59655 Villeneuve-d'Ascq Cédex, France

H. van Halbeek, J. H. G. M. Mutsaers, and J. F. G. Vliegenthart,
Bioorganisch-Chemisch Laboratorium, Rijksuniversiteit Utrecht, Croesestraat 79, NL-3522-AD Utrecht, The Netherlands