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Separation of the Complex Asparagine-Linked Oligosaccharides of the Glycoprotein Fetuin and Elucidation of Three Triantennary Structures Having Sialic Acids Linked Only to Galactose Residues†

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ABSTRACT: Asparagine-linked oligosaccharides of the glycoprotein fetuin were isolated as reducing oligosaccharides after hydrazinolysis/re-N-acetylation/mild acid treatment of the Pronase-digested glycoprotein. The sialylated oligosaccharides were separated by high-performance liquid chromatography in two different systems, which resulted in greater than 35 fractions, comprising di-, tri-, tetra-, and pentasialylated oligosaccharides. The major components were isomeric structures comprising the tri- and tetrasialylated fractions. In this and the accompanying paper (Cumming et al., 1989), the structures of 10 of the major components of the tri-, tetra-, and pentasialylated oligosaccharide fractions are described. Separation protocols and three isolated structures having sialic acid linked only to galactose are presented in this paper.

Oligosaccharides of the glycoprotein fetuin have been investigated for over 25 years (Spiro, 1962, 1964). As such, attempts to elucidate their structures have spanned virtually the entire time period of glycoprotein structural research and have generally reflected the history of technological advances achieved during development of the field. Initial investigations were performed with glycopeptide mixtures of proteolytically treated fetuin. Spiro and colleagues have provided good evidence for the structures of the major (Spiro & Bhoyroo, 1974) and minor (Edge & Spiro, 1987) O-linked oligosaccharides of fetuin, after release of the oligosaccharides from the peptide by an alkaline β-elimination. They also elucidated the general features of the N-linked structures, i.e., a branched oligosaccharide having Man and GlcNAc residues in the core region, with peripheral GlcNAc, Gal, and NeuAc residues extending from the core (Spiro, 1964).

Two conflicting reports of the Asn-linked structures appeared later (Nilsson et al., 1979; Baenziger & Fiete, 1979). Again, glycopeptides were prepared from the intact protein; no reports were made of successful subfractionation of the Asn-linked oligosaccharides in these papers, possibly due to the difficulties in separating mixtures of glycopeptides that vary in both the oligosaccharide and peptide components. At this time, neither high-field NMR[1] (Dorland et al., 1977; Strecker et al., 1977; Carver & Grey, 1981) nor HPLC was in common use for oligosaccharide structural determination.

More recently, it has been demonstrated that heterogeneity exists in the Asn-linked structures of fetuin, particularly in the presence of a Galβ1→3GlcNAc in some of the oligosaccharides (Berman, 1986; Townsend et al., 1986; Berman et al., 1988). These studies were performed with mixtures of glycopeptides, and results were deduced from the data of a mixture of oligosaccharide structures. Of importance, Takasaki and Kobata (1986) employed hydrazinolysis to liberate

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[1] Abbreviations: NMR, nuclear magnetic resonance spectroscopy; HPLC, high-performance liquid chromatography; DEAE, 2-(diethylamino)ethyl; Tris, tris(hydroxymethyl)aminomethane; NOE, nuclear Overhauser enhancement.

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oligosaccharides from the peptide chain, thereby enabling structures to be separated solely on the nature of the carbohydrate moiety. They proposed two sialylated structures having either three or four sialic acids. NMR spectra of their structures were not collected.

In our efforts to provide solid NMR evidence for the individual sialylated structures of fetuin, we utilized a modified hydrazinolysis procedure (Bendiak & Cumming, 1985, 1986) to produce reducing oligosaccharides derived from a mixture of fetuin glycopeptides. We report here and in the accompanying paper (Cumming et al., 1989) that fetuin contains a number of isomeric Asn-linked oligosaccharide structures. We have isolated and propose structures for seven trisialylated oligosaccharides, two tetrasialylated structures, and one pentasialylated oligosaccharide present on fetuin. Emphasis is placed on the crucial role of high-field NMR in the evaluation of *isomeric* purity of oligosaccharides and on the importance of using multiple HPLC columns in the purification of individual oligosaccharides from complex mixtures of isomers. In this paper, separation protocols and elucidation of the structures having sialic acid linked only to galactose are presented.

Materials and Methods

Pronase from *Streptomyces griseus* and fetuin were purchased from Calbiochem-Behring. DEAE-Sephacel (bead size 40–150 μm) and Sephadex G-15 and G-25 were from Pharmacia. Bio-Gel P2 was from Bio-Rad. Tris, ultrapure, was from Schwartz/Mann. Anhydrous hydrazine was purchased from Aldrich. Deuterium oxide of 99.8 and 99.96 atom % was from Merck Sharp & Dohme and that of 99.996 atom % was obtained from Aldrich. HPLC-grade acetonitrile was obtained from Fisher Scientific.

Preparation of Glycopeptides. Fetuin (25 g) was dissolved in 100 mL of 2 mM $CaCl_2$, 0.02% NaN_3, and 0.1 M Tris-HCl, pH 8.0. The solution was warmed to 37 °C, and Pronase (250 mg) present in the same buffer at 37 °C was added (10 mL), along with a few drops of toluene. Two subsequent 250-mg quantities of Pronase dissolved in 10 mL of the above buffer were added at 24-h intervals, after the pH was adjusted to 8.0. The digest was desalted on a Sephadex G-25 column (5.5 × 85 cm) in two batches. Fractions (15 mL) were monitored for hexose by a small-scale phenol–sulfuric acid assay (Kobata, 1972), and hexose-containing fractions were pooled, lyophilized, and redigested as above, with three additions of Pronase. The sample was again desalted on Sephadex G-25, as above.

Attempts were made at this point to separate glycopeptides by DEAE-Sephacel chromatography and HPLC. However, these separations were poor and revealed an extremely complex mixture of glycopeptides, as assessed by ^{1}H NMR of fractions (data not shown). It was noted that several fractions showed unusual chemical shifts in the regions of the spectra containing the sialic acid H-3a/H-3e signals. Since these unusual chemical shifts suggested the presence of novel structures, these fractions were pooled. To permit separations to be achieved solely on the nature of the carbohydrate moiety, glycopeptides were treated further with hydrazine, to isolate reducing oligosaccharides.

Preparation of Reducing Oligosaccharides by Hydrazinolysis. Glycopeptides were lyophilized in a vacuum hydrolysis tube (Pierce) and dried over P_2O_5 for 3 days in a vacuum desiccator. Anhydrous hydrazine was transferred in a dry glovebox under argon as previously described (Bendiak & Cumming, 1985, 1986), and the sample was heated to 100 °C for 30 h. The sample was frozen and lyophilized (caution: have ice in the trap!) to remove hydrazine. Re-N-acetylation was carried out in saturated $NaHCO_3$, having excess solid $NaHCO_3$, with acetic anhydride, as previously described (Bendiak & Cumming, 1985, 1986). The product was desalted twice on a Sephadex G-15 column (2.5 × 85 cm), monitoring for hexose being with the small-scale phenol–sulfuric acid test. The oligosaccharide was then treated with 0.1 N HCl at 35 °C for 1 h to remove the β-acetohydrazide at the reducing end of the oligosaccharides and neutralized immediately with solid $NaHCO_3$. The mild acid treatment at 35 °C also removes 3% of the NeuAc residues from oligosaccharides (Bendiak & Cumming, 1986). The product was desalted again on the same Sephadex G-15 column, above, and the phenol–sulfuric-positive peak (Kobata, 1972) was lyophilized. A sample was monitored for the presence of the α,β reducing GlcNAc residue by ^{1}H NMR, which confirmed the H-1 of the α-GlcNAc at $\delta = 5.192$ and the characteristic *N*-acetyl methyl proton signal at $\delta = 2.038$ (Bendiak & Cumming, 1986).

Separation of Reducing Oligosaccharides. (A) DEAE-Sephacel. The oligosaccharides were loaded on a DEAE-Sephacel column (2.5 × 20 cm), equilibrated in 2 mM Tris-HCl, pH 7.0, containing 0.02% NaN_3. The column was washed with 2 mM Tris-HCl, pH 7.0 (10 column volumes), followed by a gradient of 0–30 mM NaCl in the same buffer to elute disialylated oligosaccharides. A gradient from 0 to 400 mM NaCl in the same buffer was then applied. Fractions (8 mL) were collected, and aliquots (100 μL) were assayed by the small-scale phenol–sulfuric acid test (Kobata, 1972). Pools were designated disialylated (Di-S), trisialylated (Tri-S), tetrasialylated (Tetra-S), and pentasialylated (Penta-S). Peaks were desalted on a Sephadex G-15 column (2.5 × 85 cm). Tetra-S and Penta-S pools were rerun on the same DEAE-Sephacel column, elution being with a more shallow gradient from 0 to 200 mM NaCl, in 2 mM Tris-HCl, pH 7.0. These samples were again desalted on Sephadex G-15, as above. All samples were examined by ^{1}H NMR at this stage. Fractions were mixtures, as judged by the nonintegral ratios of signals in the anomeric region and the broadness of certain signals due to partially overlapping resonances. Therefore, further separations were necessary.

(B) HPLC: Aminopropyl–Silica. HPLC separations were carried out on two Waters systems, having variable-wavelength detectors (Model 490). Each of the peaks from the DEAE-Sephacel chromatography were further resolved on an aminopropyl–silica HPLC column (Alltech magnum, 1.0 × 25 cm, 10-μm particle size), absorbance being measured at 195 nm. The column was chromatographed at 1.5 mL/min, with varying mixtures of the following two solutions: A, 65% acetonitrile/35% water (v/v); B, 0.15 M hydrogen potassium phosphate buffer, pH 5.4, prepared by mixing 0.15 M H_2KPO_4 with 0.15 M HK_2PO_4 to pH 5.4. Approximately 2 μmol/batch was loaded on the aminopropyl–silica column and chromatographed for the first run with 65% A/35% B (v/v). Eluted peaks were collected manually; samples were lyophilized and loaded on a Sephadex G-15 column (1.5 × 50 cm) to desalt. The Di-S fractions (Figure 2a) were not chromatographed a second time on this column and are the topic of ongoing investigations. Peaks 2–5 of the Tri-S fraction (Figure 2b) were rerun at concentrations ranging from 70% A/30% B (v/v) to 80% A/20% B (v/v), to eliminate leading and trailing shoulders. The five peaks of the Tetra-S fraction (Figure 2c) were rerun at concentrations of 68% A/32% B (v/v). Peaks 3–5 of the Penta-S fraction were rerun at 65% A/35% B (v/v). Eluted fractions were individually collected, lyophilized, and desalted on Sephadex G-15, as described above.

Fractions were examined by ^{1}H NMR at this stage. Tetra-S 3 and 5 and Penta-S 6 were the only fractions that were

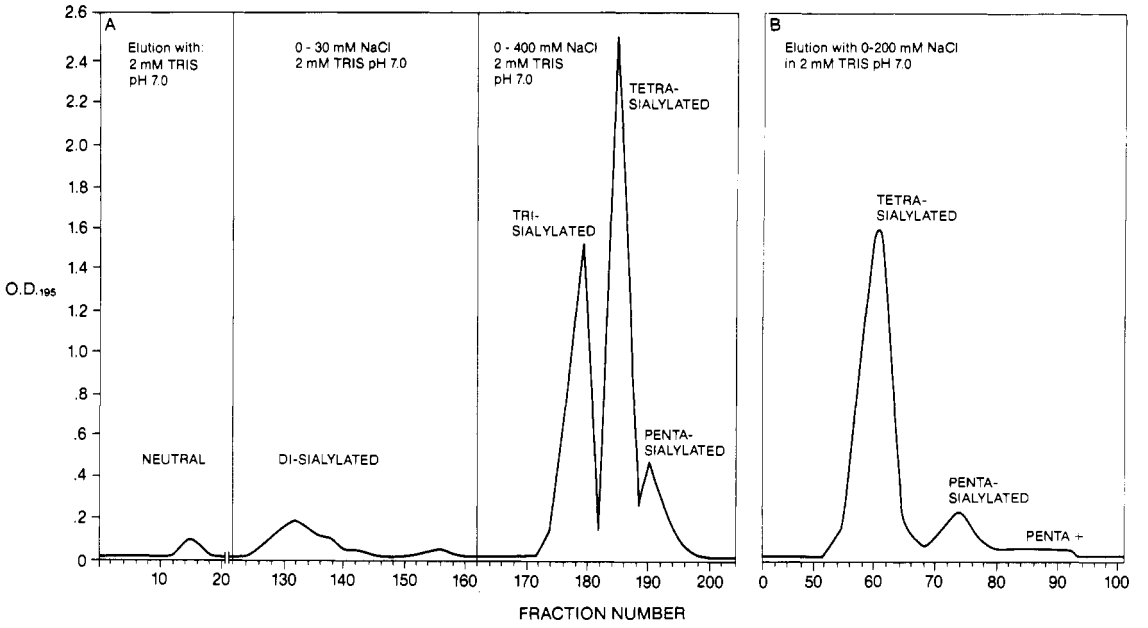

FIGURE 1: Chromatography of fetuin oligosaccharides on DEAE-Sephacel. (A) Chromatography was carried out on a 2.5 × 20 cm column, equilibrated in 2 mM Tris-HCl, pH 7.0, with an NaCl gradient of 0–30 mM followed by elution with a 0–400 mM NaCl gradient, both carried out in the same buffer. Fractions (8 mL) were monitored for hexose by a small-scale phenol–sulfuric acid test (Kobata, 1972). Pooled fractions (disialylated, trisialylated, tetrasialylated, and pentasialylated) are indicated. (B) Tetrasialylated and pentasialylated fractions, after desalting, were chromatographed a second time, with a 0–200 mM NaCl gradient in 2 mM Tris-HCl, pH 7.0. The effluent was monitored for hexose by the small-scale phenol–sulfuric acid test.

sufficiently pure to deduce their structures, as judged by anomeric proton integrations. Other peaks required additional separation.

(*C*) *HPLC: Glyco-PAK DEAE.* Most of the desalted peaks from the aminopropyl–silica column were chromatographed on a prototype polymeric anion-exchange column, Glyco-PAK DEAE (Waters, 0.75 × 7.5 cm, 10-μm particle size). Chromatography was carried out isocratically at 1.0 mL/min with varying concentrations of sodium dihydrogen phosphate buffer, brought to pH 5.4 with NaOH, detection being at 200 nm. The column easily separates oligosaccharides having different charge/mass ratios; even within a particular class, such as the trisialylated fraction, we observed resolution of isomeric structures with identical masses and charge/mass ratios. The six samples of trisialylated structures (Tri-S 1–6) from chromatography on the aminopropyl–silica column (Figure 2b) were separately loaded onto the Glyco-PAK DEAE column. Approximately 200–300 nmol of each sample was loaded per batch, and peaks from repeated batches were pooled. Tri-S 2 and 5 were eluted with 15 mM phosphate buffer, above (pH 5.4), and Tri-S 1, 3, 4, and 6 were eluted with 12.5 mM phosphate buffer. Peaks were desalted on a Sephadex G-15 column (1.6 × 95 cm); compounds were detected as a peak at the void volume by monitoring fractions at 200 nm. Oligosaccharides were rerun on the Glyco-PAK DEAE column, elution being with 12.5 mM phosphate buffer; small shoulders present on either side of main peaks were eliminated. Oligosaccharides Tri-S 2A and Tri-S 2B were rechromatographed an additional time, 12.5 mM phosphate buffer being used, because they were only partially resolved. Even after this third run, Tri-S 2B contained about 20% Tri-S 2A but was pure enough to identify the major component by NMR. All oligosaccharides were desalted on Sephadex G-15, as above. Tetra-S 2 (Figure 2c) was chromatographed on the Glyco-PAK DEAE column with 28 mM sodium phosphate buffer, pH 5.4. Individual pools were desalted on Sephadex G-15, rerun on Glyco-PAK DEAE under the same conditions, and again desalted. Penta-S 1, 3, and 4 (Figure 2d) were chromatographed on the Glyco-PAK DEAE column with 50 mM sodium phosphate buffer, pH 5.4, and desalted on Sephadex G-15; individual peaks were rerun on Glyco-PAK DEAE under identical conditions and again desalted. Although several of the oligosaccharides were pure, or relatively pure at this stage, as judged by ^{1}H NMR, some were still mixtures, and further separation on additional columns is the topic of ongoing studies.

500-MHz ^{1}H NMR Spectrum Acquisition. Samples were desalted on Sephadex G-15 and exchanged twice in 99.8% ^2H_2O and dissolved in 99.96% ^2H_2O for transfer to NMR tubes. The samples were then lyophilized in the NMR tubes and transferred under argon to a dry box. Samples were dissolved in either 99.96% or 99.996% ^2H_2O for accumulation of spectra. One-dimensional ^{1}H spectra were collected on a Bruker AM500 spectrometer at 27 °C. Chemical shifts were recorded by reference to internal acetone (δ = 2.225, with an accuracy to ±0.002 ppm). Spectra were averages of 512–16384 transients with 32000 real/imaginary points. Averaged transients were processed with base-line correction and apodized with either exponential or Gaussian multiplication before Fourier transformation. When necessary, polynomial base-line correction was applied to the final spectrum.

Desialylation of Oligosaccharides. Desialylation of oligosaccharides was carried out as described by Spiro (1966). Sulfate (due to sulfuric acid used in the hydrolysis) and released sialic acid were removed on Dowex AG 1-X8 (acetate form) as described (Spiro, 1966).

Results

Separation of Oligosaccharides. The elution profile of hydrazine-released fetuin oligosaccharides on a DEAE-Sephacel column is shown in Figure 1. Fractions were pooled as indicated; four major pools (Di-S, Tri-S, Tetra-S, and Penta-S) were desalted on Sephadex G-15 and subjected to chromatography on aminopropyl–silica. A small pool (Penta +) was not further chromatographed. The resultant separation of fraction Di-S is shown in Figure 2a. Five major compo-

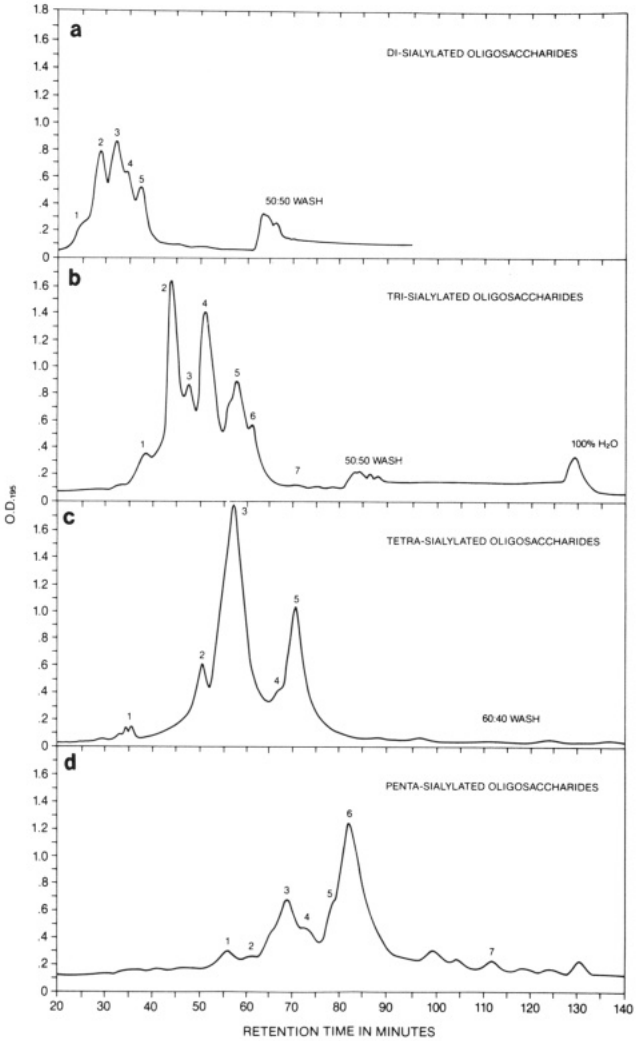

FIGURE 2: Oligosaccharide fractionation by HPLC on an Alltech aminopropyl–silica column (1.0 × 25 cm). Fractions from the DEAE-Sephacel column (Figure 1) were further separated with a mobile phase comprised of 65% A/35% B, where A was 65% acetonitrile–35% water and B was 0.15 M hydrogen potassium phosphate buffer, pH 5.4. Shown are elution profiles of oligosaccharide fractions from Figure 1: (a) the disialylated (Di-S) fraction, (b) the trisialylated (Tri-S) fraction, (c) the tetrasialylated (Tetra-S) fraction, and (d) the pentasialylated (Penta-S) fraction. Small amounts of structures were recovered in washes having a higher phosphate/acetonitrile ratio (A:B ratios of washes are shown in each chromatographic profile).

nents (Di-S 1–5) were isolated and desalted. Similarly, Tri-S (Figure 2b), Tetra-S (Figure 2c) and Penta-S (Figure 2d) were chromatographed on the aminopropyl column, yielding seven, five, and seven fractions, respectively. Yields of oligosaccharides off the aminopropyl–silica column were low (approximately 50%). However, the column does not appear to selectively diminish particular species from the mixture. ^{1}H NMR at this stage revealed different structures and mixtures of structures in each peak eluted. Of the eluted peaks, only Tetra-S 3 and 5 and Penta-S 6 represented relatively homogeneous compounds at this stage [see Cumming et al. (1989) for NMR spectra].

Peaks eluted from the aminopropyl–silica column were then individually chromatographed on the anion-exchange column Glyco-PAK DEAE. Elution profiles of Tri-S 1-6 are shown in Figure 3a–f. Each peak from the aminopropyl–silica column separated into four or five components on the Glyco-PAK DEAE column. Many of these peaks were rechromatographed after desalting. Recoveries off the Glyco-PAK DEAE column, including the subsequent desalting step, were

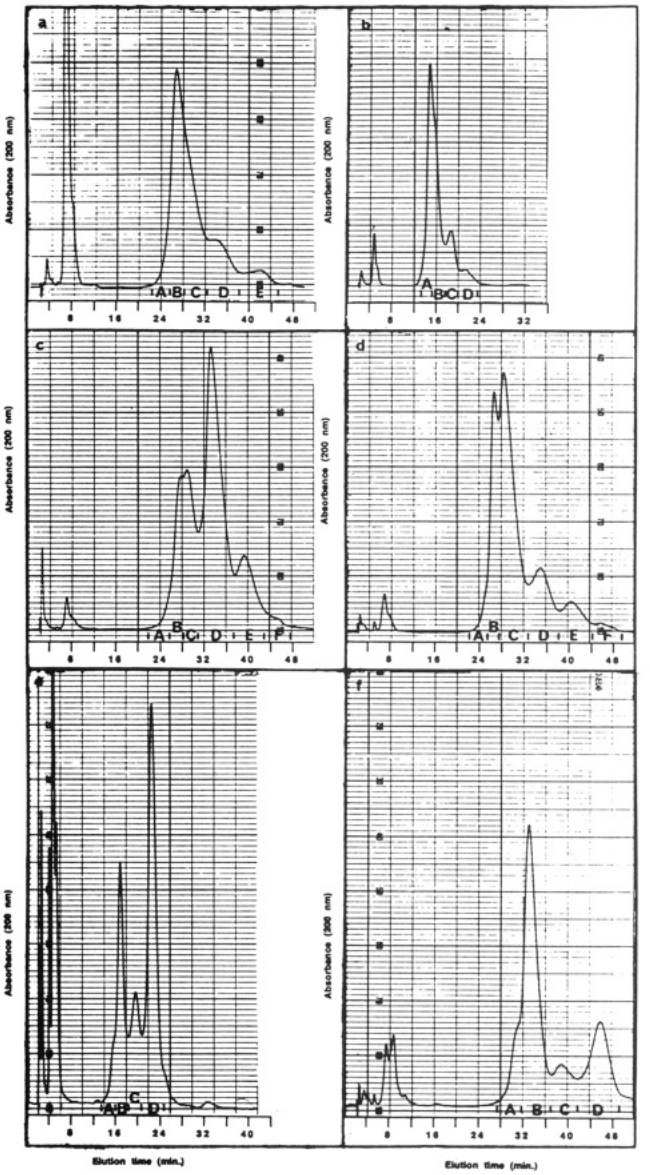

FIGURE 3: HPLC of trisialylated fetuin oligosaccharide fractions on Glyco-PAK DEAE. Six trisialylated fractions (Tri-S 1–6), initially isolated from the aminopropyl–silica column (Figure 2b), were chromatographed on a Glyco-PAK DEAE column (0.75 × 7.5 cm) at various concentrations of sodium hydrogen phosphate buffer, pH 5.4, while elution was being monitored by UV absorbance at 200 nm. Fraction number from the aminopropyl–silica chromatography (Figure 2b) and sodium hydrogen phosphate concentration for each chromatography were as follows: (a) fraction 1, 12.5 mM; (b) fraction 2, 15 mM; (c) fraction 3, 12.5 mM;(d) fraction 4, 12.5 mM; (e) fraction 5, 15 mM; (f) fraction 6, 12.5 mM. Peaks were collected as indicated, desalted on a Sephadex G-15 column, and rerun on the Glyco-PAK column at least once in 12.5 mM phosphate buffer to eliminate small leading and trailing peaks.

greater than 80%. Many of the oligosaccharides at this stage were relatively pure, as evaluated by ^{1}H NMR. However, some remained mixtures. Similarly, the elution profile of Tetra-S 2 and Penta-S 1, 3, and 4 on Glyco-PAK DEAE are shown in Figure 4 and Figure 5a–c, respectively. Peaks were pooled as indicated, desalted, and rerun, finally desalting prior to NMR. The estimated quantities of purified oligosaccharides are shown in Table I and are based on integrated areas under individual peaks of purified compounds. As mentioned, those glycopeptide fractions of fetuin which showed unusual chemical shifts of the NeuAc H-3a/H-3e signals and therefore represented novel structures were pooled for hydrazine treatment. Oligosaccharides of fetuin having standard NeuAc H-3a/H-3e

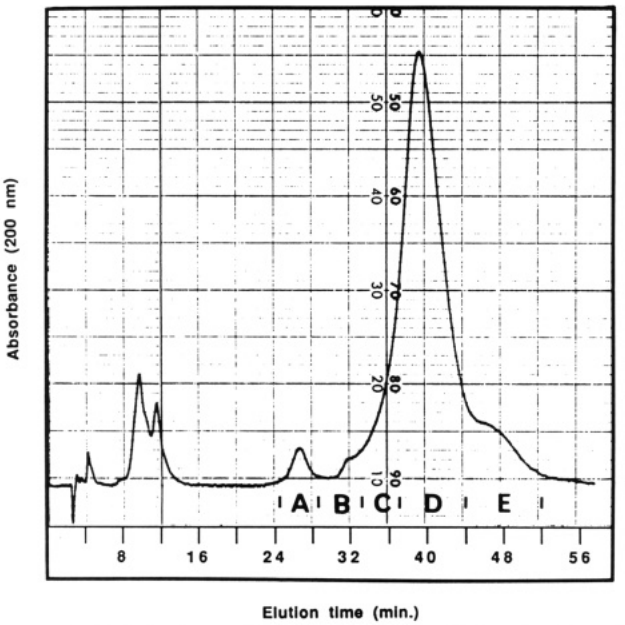

FIGURE 4: HPLC of tetrasialylated oligosaccharide fraction Tetra-S 2 on Glyco-PAK DEAE. Fraction Tetra-S 2, from Figure 2c, was chromatographed on a Glyco-PAK DEAE column (0.75 × 7.5 cm) with 28 mM sodium hydrogen phosphate buffer, pH 5.4. Eluent was monitored by measuring absorbance at 200 nm. Peaks were collected as indicated and desalted; major fractions (A, D, and E) were rerun on the Glyco-PAK column at least once, to eliminate small leading and trailing peaks.

Table I: Relative Abundance of Purified Oligosaccharides[a]

oligosaccharide	% total	% trisialyl fraction	% tetrasialyl fraction	% pentasialyl fraction
Tri-S 2A	5.5	16.4		
Tri-S 2B	2.3	6.9		
Tri-S 3D	2.0	6.0		
Tri-S 4B	2.3	6.9		
Tri-S 5C	1.0	3.0		
Tri-S 5D	3.5	10.5		
Tri-S 6B	1.4	4.2		
Tetra-S 3	24.3		54.1	
Tetra-S 5	10.3		22.8	
Penta-S 6	4.3			44.0

[a] Relative abundance is based on integration of chromatographic peaks. Oligosaccharides were prepared from those glycopeptide fractions of fetuin which showed unusual chemical shifts of the NeuAc H-3a/H-3e signals and therefore contained novel structures. Consequently, purified oligosaccharides of fetuin having only Galβ1→4GlcNAc linkages in the branches (Tri-S 2A and 4B) are probably underestimated in this table.

chemical shifts (i.e., having only Galβ1→4GlcNAc linkages in the branches) are probably underestimated in Table I.

Nevertheless, on the basis of our previous studies with hydrazine treatment of total fetuin glycopeptides (Bendiak & Cumming, 1986), we estimate the Galβ1→3GlcNAc linkage to comprise 10–15% of the total galactose of Asn-linked oligosaccharides. As these oligosaccharides are primarily triantennary, this corresponds to 30–45% of the total Asn-linked oligosaccharides having one Galβ1→3GlcNAc branch. This is in good agreement with the data of Berman (1986), where 12% of the total galactose was determined to be in β1→3 linkage and therefore about 36% of the Asn-linked oligosaccharides contained one such branch. In addition, in the paper by Townsend et al. (1986), the three individual glycosylation sites of fetuin were purified, and on the average, 35–40% of the Asn-linked oligosaccharides contained one Galβ1→3GlcNAc branch.

Structures of Oligosaccharides Having Sialic Acid Linked Only to Galactose. In this and the accompanying paper, much of the evidence for oligosaccharide structures and, in particular, the branch location of specific sialic acid residues is based on 500-MHz ^{1}H NMR data. In oligosaccharides that contain a set of similar branches, such as the sialylated structures described herein (Figure 6), the assignment of specific signals to a particular branch should be based on prior knowledge of the assignments of a closely related series of structures. In a series of sialylated triantennary glycopeptides described previously (Joziasse et al., 1987), signals could be assigned to individual branches by comparing a set of products isolated after the sequential action of an α2→6 sialyltransferase. Perturbations in the chemical shifts of signals associated with

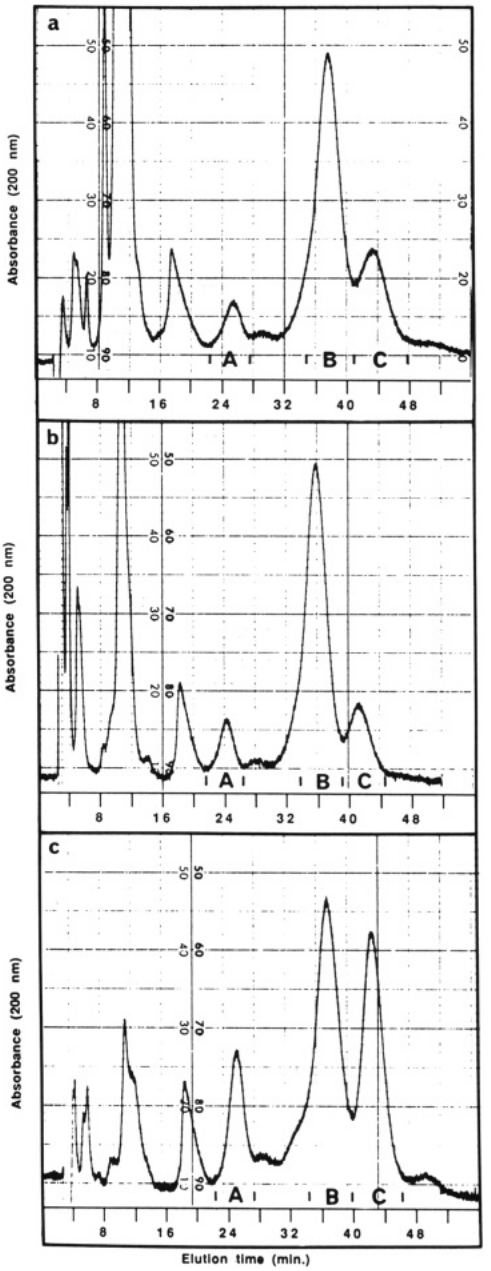

FIGURE 5: HPLC of pentasialylated oligosaccharide fractions on Glyco-PAK DEAE. Fractions 1, 3, and 4 (from Figure 2d) were chromatographed on Glyco-PAK DEAE (0.75 × 7.5 cm) with 50 mM sodium hydrogen phosphate buffer, pH 5.4, as the mobile phase. Eluent was monitored by measuring absorbance at 200 nm. Shown are elution profiles of chromatograms of (a) fraction 1 (Figure 2d), (b) fraction 3 (Figure 2d), and (c) fraction 4 (Figure 2d). Peaks were collected as shown, and major carbohydrate-containing fractions were rechromatographed on the Glyco-PAK DEAE column to eliminate small leading and trailing peaks.

Table II: Chemical Shifts of Structural Reporter Protons of Oligosaccharides from Fetuin Having Sialic Acid Linked Only to Galactose

	oligosaccharide and schematic structure			
	N6G4Gn2M 6 M4Gn4Gnβ-N-Asn 3 N6G4Gn2M 4 N6G4Gn	N6G4Gn2M 6 M4Gn4Gn(α,β) 3 N6G4Gn2M 4 N3G4Gn	N3G4Gn2M 6 M4Gn4Gn(α,β) 3 N6G4Gn2M 4 N3G4Gn	N3G4Gn2M 6 M4Gn4Gn(α,β) 3 N6G4Gn2M 4 N3G3Gn
proton	GP3-TS[a]	Tri-S 4B	Tri-S 2A	Tri-S 2B
H-1 of				
1	5.060	5.192 (α)	5.192 (α)	5.192 (α)
2	4.615	4.61 (α)[b]	4.612 (α)	4.61 (α)[b]
3	4.76	~4.76[c]	~4.76[c]	~4.76[c]
4	5.133	5.130	5.131	5.132
4′	4.936	4.938	4.910	4.907
5	4.594	4.595	4.595	4.594
5′	4.602	4.604	4.574	4.573
6	4.439	4.445	4.444	4.441
6′	4.448	4.448	4.548	4.550
7	4.571	4.549	4.551	4.573
8	4.440	4.549	4.548	4.510
H-2 of				
3	4.220	4.219	4.217	4.218
4	4.225	4.219	4.217	4.218
4′	4.116	4.116	4.115	4.116
H-3 of				
4	nd	4.045	4.044	4.044
6′	nd	<4.0	4.119	4.118
8	nd	4.118	4.119	4.092
H-3a of				
N	1.717	1.720	1.720	1.720
N′	1.717	1.720	1.802	1.803
N*	1.706	1.803	1.802	1.787
H-3e of				
N	2.670	2.669	2.668	2.668
N′	2.674	2.674	2.758	2.759
N*	2.670	2.757	2.758	2.759
NAc of				
1	2.008	2.038	2.038	2.039
2	2.082	2.083	2.082	2.082
5	2.069	2.066	2.066	2.067
5′	2.065	2.066	2.044	2.044
7	2.101	2.074	2.074	2.067
N	2.030	2.031	2.031	2.030
N′	2.030	2.031	2.031	2.030
N*	2.030	2.031	2.031	2.030

[a] From Joziasse et al. (1987). Signals of this glycopeptide are shown here for comparative purposes (nd refers to signals not reported in that paper). [b] Chemical shift only assigned to two decimal places due to spectral overlap. [c] Approximate shift only due to overlapping $^1HO^2H$ signal.

the residues of just one branch were observed as sialic acid was added to that particular branch. Therefore, signals associated with each branch could be established in the completely sialylated and in the desialylated structures. The importance of making assignments on the basis of a series of oligosaccharide structures is emphasized because the assignments of the galactose H-1 signals in the desialylated, triantennary glycopeptide described by Joziasse et al. (1987) were corrected from their previous assignments (Vliegenthart et al., 1983). We have verified the corrected assignments (Cumming et al., 1989) and stress the point that, whenever possible, assignments of signals to specific branches should be confirmed by appropriate NOE experiments.

Tri-S 4B. The proposed structure of the major component of fraction Tri-S 4B (Figure 3d) is shown in Figure 6. The 500-MHz ^1H NMR spectrum of this compound is shown in Figure 7a, and its proton assignments are in Table II. It is identical with a structure isolated from a patient having sialidosis (Vliegenthart et al., 1983) except, instead of having a single GlcNAc at the reducing end, this structure bears a di-*N*-acetylchitobiose unit. This core disaccharide in all compounds is identified by the standard chemical shifts for the H-1 doublet of the reducing α-GlcNAc (δ = 5.192) and the *N*-acetyl singlet (δ = 2.038; Bendiak & Cumming, 1986). The H-1 signal for the reducing β-GlcNAc is near δ = 4.7 and is often partially obscured by the $^1HO^2H$ peak at 27 °C. The H-1 signals of GlcNAc residue 2 appear as two doublets of differing intensity near δ = 4.61 and 4.60, due to different induced magnetic environments caused by the two anomers of the reducing GlcNAc. Often, the GlcNAc 2 H-1 signal is partially obscured by other GlcNAc H-1 signals, which have a similar chemical shift.

The structure is readily characterized as a triantennary N-linked type of oligosaccharide having branch substitutions on the 2-position of the α1→6-linked mannose and on the 2- and 4-positions of the α1→3-linked mannose. This conclusion is based on the unique H-1/H-2 patterns of the mannose residues and the downfield position (δ = 4.045) of the H-3 quadruplet of Man 4 (Fournet et al., 1978; Carver & Grey, 1981; Vliegenthart et al., 1983). All three structures reported in this paper (Figure 6) have the same substitutions on the mannoses, on the basis of these unique Man H-1/H-2 chemical shifts and the chemical shift of the H-3 of Man 4.

In structure Tri-S 4B, two of the branches are NeuAcα2→6Galβ1→4GlcNAcβ1→R, and one is NeuAcα2→3Galβ1→4GlcNAcβ1→R. These assignments are

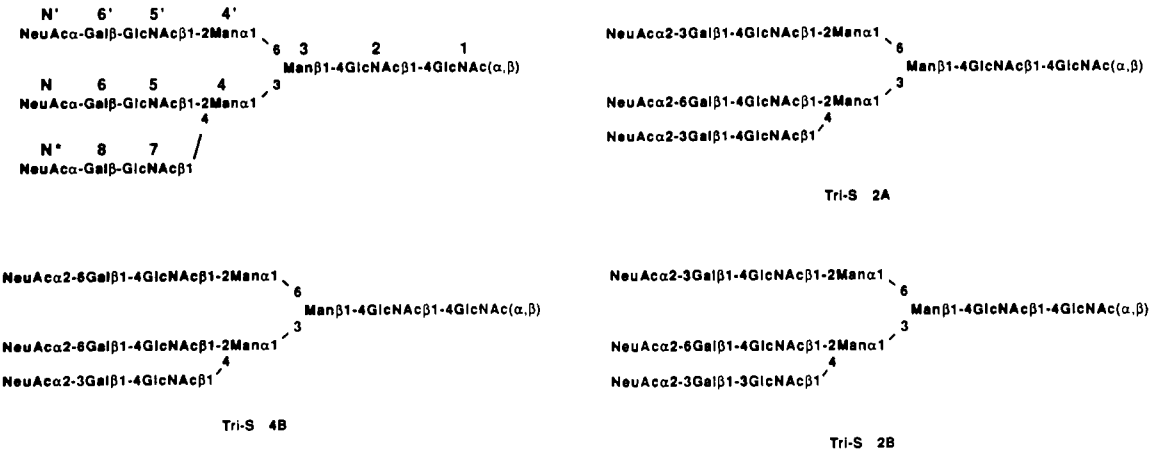

FIGURE 6: Standard residue numbering system is shown in the generalized structure at the upper left [from Vliegenthart et al. (1983)]. The proposed structures of three oligosaccharides isolated from fetuin having NeuAc linked only to galactose residues (Tri-S 2A, 2B, and 4B) are presented.

based on the relative quantities and distinct chemical shifts of the NeuAc H-3a/H-3e and NAc proton signals, Gal H-1 and H-3 signals, and GlcNAc H-1 and NAc signals. The specific localization of the NeuAcα2→3Galβ1→4GlcNAcβ1→R arm to the 4-position of the α1→3-linked mannose can be made by a comparison to the similar triantennary glycopeptide which bears all NeuAc residues in α2→6 linkage to galactose (Joziasse et al., 1987). The chemical shifts of that structure are presented, for comparison, in Table II. Signals associated with residues of the Galβ1→4GlcNAcβ1→4Man branch (GlcNAc 7, Gal 8, and NeuAc N*) were perturbed significantly. Since major chemical shift perturbations were restricted to this branch and because of the close spectral relationship of Tri-S 4B to the oligosaccharide previously described from a sialidosis patient by Vliegenthart et al. (1983), the structure of this oligosaccharide is proposed to be that shown in Figure 6.

Tri-S 2A. The proposed structure of the major component of fraction Tri-S 2A (Figure 3b) is shown in Figure 6, its ^{1}H NMR spectrum is shown in Figure 7b, and its proton assignments are shown in Table II. The spectrum of the desialylated molecule is presented in the accompanying paper (Cumming et al., 1989), which established the molecule as a triantennary oligosaccharide bearing galactose residues only in β1→4 linkage to GlcNAc. It is closely related to oligosaccharide Tri-S 4B (above), differing only in that the linkage of the NeuAc on the branch attached to Man 4′ is α2→3 instead of α2→6. This is evidenced by the major shifts of all reporter signals assigned to residues of the Manα1→6 branch, including Man 4′ H-1 (from δ = 4.938 to δ = 4.910), GlcNAc 5′ H-1 (from δ = 4.604 to δ = 4.574) and *N*-acetyl protons (from δ = 2.066 to δ = 2.044), Gal 6′ H-1 (from δ = 4.448 to δ = 4.548), and NeuAc N′ H-3a/H-3e (from δ = 1.720/2.674 to δ = 1.802/2.758). No significant shift perturbations of signals assigned to residues of the branches attached to the Man α1→3 arm occur. Specific chemical shift effects of NeuAc either α2→3 linked or α2→6 linked to a Galβ1-4GlcNAc terminus of Asn-linked carbohydrate structures have been well documented [for a review, see Vliegenthart et al. (1983)]. The structure of Tri-S 2A was actually first proposed by Nilsson et al. (1979) to be the single component of Asn-linked species in fetuin.

Tri-S 2B. The proposed structure of Tri-S 2B is shown in Figure 6, its ^{1}H NMR spectrum is shown in Figure 7c, and its proton assignments are shown in Table II. It was obtained as the more-retained shoulder of peak Tri-S 2A (Figure 3b) and was rerun twice on the Glyco-PAK DEAE column, the front one-third of the peak being eliminated each time to purify the compound in the back shoulder as much as possible. Although it contained a single major component, it also contained an estimated 20% of Tri-S 2A, as assessed by NMR. Nevertheless, a unique structure for the major component of Tri-S 2B could be assigned. The compound is related to structure Tri-S 2A, in that Tri-S 2B bears a NeuAcα2→3Galβ1→*3*GlcNacβ1→4Man instead of a NeuAcα2→3Galβ1→*4*GlcNAcβ1→4Man branch. The most evident features in the spectrum are the doublet appearing at δ = 4.510, which is assigned to Gal 8 H-1, and the N* H-3a/H-3e signals at δ = 1.787/2.759. These signals were assigned to a linear NeuAcα2→3Galβ1→3GlcNAcβ1→R sequence by comparison to the model compound NeuAcα2→3Galβ1→3GlcNAcβ1→3Galβ1→4Glc (IV^{3}NeuAc-LcOse$_4$), an oligosaccharide isolated from milk which has been previously examined by NMR and that has had its anomeric signals assigned (Bernard et al., 1984). For IV^{3}NeuAc-LcOse$_4$, the signals of the NeuAc H-3a/H-3e appear at δ = 1.786/2.759 and that of the H-1 of the Galβ1→3 to GlcNAc appears at δ = 4.508. In both compounds, the characteristic Gal H-3 quadruplet is shifted downfield out of the major proton envelope to near δ = 4.09, due to substitution with NeuAc at the 3-position. While this indicates the presence of a NeuAcα2→3Galβ1→3GlcNAcβ1→R antenna, it does not establish its linkage to an α-linked mannose.

Assignment of this specific sequence to the branch originating at the 4-position of Man 4 is based on two criteria. First, the desialylated compound revealed a ^{1}H NMR spectrum identical with that of desialylated Tetra-S 3 (Cumming et al., 1989), where the Galβ1→3GlcNAc branch was firmly established by NOE experiments to be located at the 4-position of Man residue 4. Second, a comparison between Tri-S 2A and Tri-S 2B (Table II) reveals perturbations in chemical shifts of signals assigned to residues of the 4-linked branch. Marked changes are observed in chemical shifts of signals of GlcNAc 7 (H-1 from δ = 4.551 to δ = 4.573 and NAc protons from δ = 2.074 to δ = 2.067), Gal 8 (H-1 from δ = 4.548 to δ = 4.510 and H-3 from δ = 4.119 to δ = 4.092), and NeuAc N* (H-3a from δ = 1.802 to δ = 1.787). On the other hand, chemical shift differences assigned to residues of the other two branches were negligible.

Discussion

Although the glycoprotein fetuin is readily obtained in relatively large amounts, NMR data on individual sialylated oligosaccharide structures have been slow in arriving. The

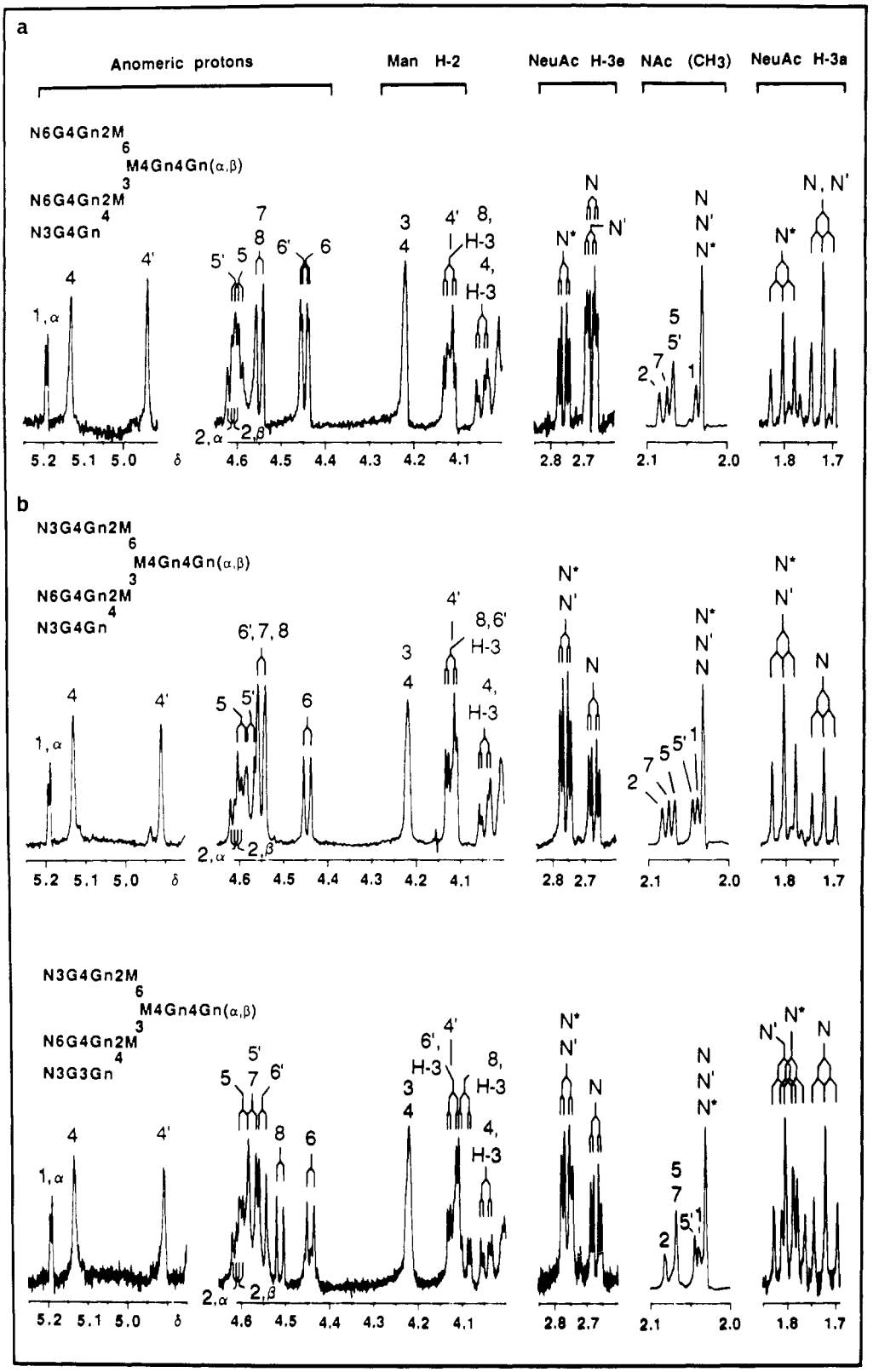

FIGURE 7: Partial 500-MHz ^{1}H NMR spectra of sialylated fetuin oligosaccharides having NeuAc residues linked only to Gal residues. Shown are the schematic structures and partial spectra of (a) Tri-S 4B, (b) Tri-S 2A, and (c) Tri-S 2B. Assigned signals are indicated, with the nomenclature of Vliegenthart et al. (1983), as shown in Figure 6. Separate sections of the spectra have been plotted with differing intensities. Therefore, signal integration, as presented, differs from section to section. Schematic structures are shown with each spectrum; for a complete description of anomericity and type of linkage, see the oligosaccharides as presented in Figure 6.

mixture of asparagine-linked oligosaccharides present on fetuin exemplifies a classic and well-known problem in glycoprotein research, that of heterogeneity of the carbohydrate side chains. When such heterogeneity is due to varying molecular mass or different charge/mass ratios of oligosaccharides, separations are achieved with relative ease. For example, the four classes of oligosaccharides present on fetuin having different charge/mass ratios (di-, tri-, tetra-, and pentasialyl) are readily separated by open-column ion-exchange chromatography (Figure 1).

However, the oligosaccharides of fetuin represent a case in which considerable *isomeric* heterogeneity exists. In the trisialylated fraction, for example (peak Tri-S, Figure 1), at least seven oligosaccharides were present, all of which had an identical mass and an identical charge/mass ratio. These structures were separable by HPLC, and this paper describes methods for separation of these closely related oligosaccharides which we think will be useful for the isolation of sialylated oligosaccharides from a number of sources. The two HPLC columns employed were an Alltech aminopropyl-bonded, silica-based column and a Waters polymeric Glyco-PAK DEAE column. Although both columns resulted in reasonable separations of these oligomers, each column alone was incapable of providing the degree of resolution needed for purifying many of the oligosaccharides; a combination of the two columns, which appear to separate the oligosaccharides on a different basis of interaction, was required for purification of many of these isomeric structures. Even after two HPLC separations, several fractions that we analyzed by NMR remained mixtures. It is clear that multiple, high-resolution chromatographic separations will be required for the purification of many closely related oligosaccharides from natural sources; the more HPLC columns that are available which achieve these separations, the greater will be the ease in firmly establishing their structures.

In addition, the oligosaccharides of fetuin described in this and the accompanying paper illustrate an important point regarding evaluation of *isomeric* purity of chromatographic peaks. Although additional HPLC in different systems is typical for assessing purity, as shown in this paper, even additional HPLC systems might fail to separate two closely related oligosaccharide isomers. The occurrence of such isomers in nature is common, and the possibility of isomers comigrating in successive chromatography steps must be considered. How, then, can one be certain of the isomeric purity of an unknown oligosaccharide sample in a chromatographic peak? Clearly, if peaks are not evaluated for isomeric purity, further investigations by classical means risk attempting to elucidate a structure from data derived from a mixture of molecules.

In this regard, the utility of high-field NMR cannot be overemphasized. It is the only adequate tool for evaluation of isomeric purity of purportedly "pure" chromatographic fractions. As NMR is nondestructive and permits subsequent analyses to be performed, we consider it a necessary criterion for a convincing demonstration of an oligosaccharide structure. Without its use, the related sialylated oligosaccharides of fetuin described in this and the accompanying paper would have been extremely difficult to characterize.

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