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REINVESTIGATION OF THE CARBOHYDRATE CHAINS OF CALF FETUIN USING ^{13}C-N.M.R. SPECTROSCOPY*

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ABSTRACT

The triantennary structure of the *N*-linked oligosaccharide side-chain of fetuin, elucidated by a combination of ^{13}C-n.m.r. spectroscopy and enzymic degradations, accords with that reported earlier with respect to the branching pattern, but the ratio of the *N*-acetylneuraminic acid linkages to the galactose residues [α-(2→3) *vs.* α-(2→6)] was found to be 1:1, indicating structural heterogeneity of the side chains. Also, one out of nine galactosyl residues is linked to 2-acetamido-2-deoxy-β-D-glucose by a (1→3) rather than a (1→4) linkage. The chemical shifts reported are in excellent agreement with those for the intact glycoprotein. Unusual chemical shift effects lead to the conclusion that the α-NeuAc-(2→6) residues interact with other parts of the oligosaccharide side-chain. The action of β-D-galactosidase from *Aspergillus niger* on desialylated fetuin removed ~85% of the β-Gal residues (1→4)-linked to GlcNAc and 65% of the β-Gal residues (1→3)-linked to GalNAc, but none of the β-Gal residues (1→3)-linked to GlcNAc.

INTRODUCTION

Calf fetuin is an α-globulin and the major glycoprotein of fetal calf serum[1]. Investigations on the *N*-linked side-chains of fetuin showed that the three complex sugar side-chains have an identical triantennary structure[2–4]. However, two isomeric structures were proposed that differ in the attachment positions of the α-NeuAc-(2→3)-β-D-Gal-(1→4)-β-D-GlcNAc-(1→4)- antenna and the α-NeuAc-(2→6) residue. Nilsson *et al.*[3] proposed structure **1** and Baenziger and Fiete[4] proposed structure **2**.

Support for structure **1** was given by Krusius and Finne[5] who used Smith degradations to evaluate the branching pattern. On the basis of methylation analysis, Krusius *et al.*[6] determined that the majority of the galactosyl residues are 6-*O*-substituted by α-NeuAc and, in addition to the 3-substituted galactosyl

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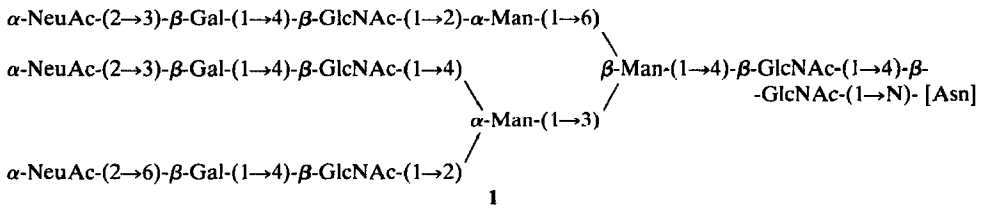

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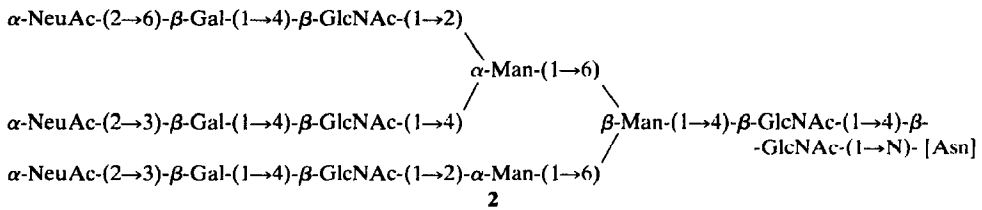

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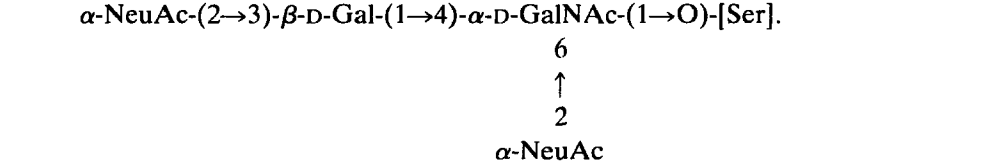
residues, there was a small proportion of terminal galactosyl groups. The latter finding is also supported by the work of Piller and Cartron[7].

Of the total sugar content of calf fetuin, ~20% is found in the form of three *O*-linked oligosaccharide units having the proposed[2,3] structures:

α-NeuAc-(2→3)-β-D-Gal-(1→4)-α-D-GalNAc-(1→O)-[THr],

α-NeuAc-(2→3)-β-D-Gal-(1→4)-α-D-GalNAc-(1→O)-[Ser],

and



N.m.r. spectroscopy is now an indispensable tool in the study of oligosaccharides and glycopeptides derived from glycoproteins[8,9], and ^{13}C-n.m.r. spectroscopy is particularly useful[9–13] for the study of intact glycoproteins; we have now applied this technique to fetuin.

EXPERIMENTAL

Materials. — Calf fetuin [types X (6.2% NeuAc) and III (5.6% NeuAc)], neuraminidase (EC 3.2.1.18) from *Cl. perfringens* (type X), and β-D-galactosidase (EC 3.2.1.23) from *Aspergillus niger* (type V) were purchased from Sigma. All other chemicals used were of analytical grade and, where needed, they were recrystallised.

Digestion of fetuin. — (*a*) *With neuraminidase.* The digestion[4] was monitored[14] and was ~95 ±8% complete after ~30 h. ^{13}C-N.m.r. analysis confirmed that not all NeuAc residues were removed and the sample was treated with more neuraminidase, resulting in an additional ~2% digestion.

(*b*) *With β-D-galactosidase.* The digestion followed the published procedure[15], using the sialic acid-free fetuin generated in (*a*). The ^{13}C-n.m.r. spectral analysis showed that ~75% of the galactose was removed after 70 h. Additional digestion did not release any more galactose.

Fetuin glycopeptides. — The published procedure[4] was employed, but the amounts of protease were increased to a total of 5%. After gel filtration on a column (43 × 3 cm) of Sephadex G-25, using 0.1M acetic acid at 35 mL/h, the material in the combined orcinol-positive fractions was redigested with half the protease used for the first digestion. Following gel filtration, the lyophilised material was eluted from a column (48 × 1.6 cm) of Dowex 50-X2 (H^+) resin with mM sodium acetate buffer (pH 3.5) at 24 mL/h. The carbohydrate containing-fraction in the void volume was collected, lyophilised, and desalted on a column (180 × 2 cm) of Bio-Gel P4 by elution with 50mM ammonium hydrogencarbonate at 6.5 mL/h (Fig. 1). Fraction *A*, which contained the *N*-linked glycopeptide part, was lyophilised and the product was rechromatographed on the above gel-filtration column.

Fraction *A* (120 mg) was eluted from a column (42 × 2 cm) of Sephadex DEAE A-25-120 with a linear gradient of ammonium hydrogencarbonate (500 mL, 0.1→0.5M, pH 7.8) at 15 mL/h. Fractions (3 mL) were collected as shown in Fig. 2.

Enzymic degradation of the N*-linked glycopeptides.* — The digestions followed known procedures[2–4]. Fraction *A* (100 mg) was digested thrice with neuraminidase, with a total of 10 units of the enzyme. After each digestion (24 h),

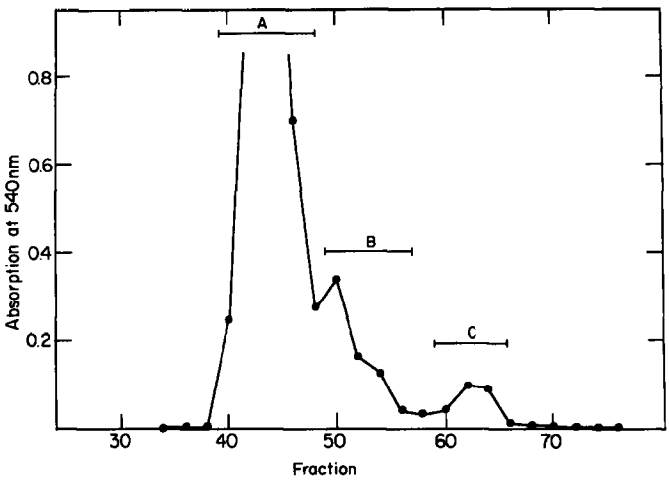


Fig. 1. Chromatography on Bio-Gel P-4 of glycopeptides from fetuin (3.5-mL fractions).

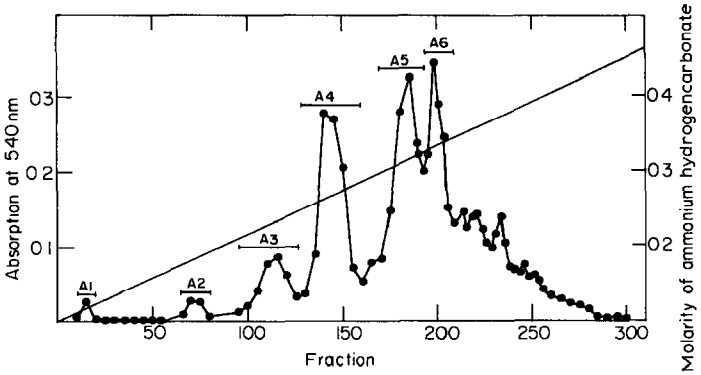


Fig. 2. Anion-exchange chromatography on DEAE A-25 of the *N*-linked glycopeptides from fetuin.

the reaction mixture was analysed by ^{13}C-n.m.r. spectroscopy and desalted on a P4 column. The β-D-galactosidase (20 units) digestion was carried out on the desialylated fraction *A* (~100 h) and then repeated with 10 enzyme units, the incubate was desalted, and the product was rechromatographed on the P4 column. Two hexose-positive fractions were eluted. The first contained the digested *N*-linked glycopeptides, and the second was identified by n.m.r. spectroscopy as a mixture of galactose and 2-acetamido-2-deoxyglucose in the ratio ~2:1.

N.m.r. spectroscopy. — All native and enzymically degraded fetuin samples were dissolved in 50mM sodium phosphate (pH ~7.0), and a denatured sample was prepared in the same buffer containing 6M urea. Both the native and the denatured samples were run as solutions in 95% D_2O, using 20-mm n.m.r. tubes, and the enzymically digested samples were run as solutions in 15% D_2O, using 10-mm n.m.r. tubes. The ^{13}C-n.m.r. spectra were obtained at 28–32° (36° for the denatured sample). All the fetuin samples were dialysed extensively before the n.m.r. spectra were recorded. The ^{13}C-n.m.r. spectra were obtained as described[16] with a line broadening of 3 Hz. Peak integrations were performed numerically using the 1180 Nicolet software. Line widths were determined by the line-fit software routine provided by Nicolet (NTCCAP). 42,000–90,000 Scans were needed to generate the final spectra with a recycle time of 3 s for the intact and denatured samples and 1 s for the enzyme-treated fetuin samples using an 85° pulse. Chemical shifts were referenced to the internal 1,4-dioxane line at 67.86 p.p.m.

The ^{13}C-n.m.r. spectra of the *N*-linked glycopeptides were obtained at 60.5, 67.9, and 90.6 MHz, and the ^{1}H-n.m.r. spectra at 240 and 360 MHz on solutions in ≥99.8% D_2O. The pH values were measured in D_2O solutions. Unless indicated, the ^{13}C-n.m.r. spectra were obtained as described above. The ^{1}H-n.m.r. spectra were obtained with a 2400-Hz spectral window, 60° pulse, 1-s recycle time, employing a solvent peak suppression routine, 16k data points, and 40–800 scans for each spectrum. The spectra were processed with 0.2–0.3-Hz line broadening. Peaks were referenced to the *N*-acetylaminomethyl group of α-NeuAc at 2.03 p.p.m. as a secondary standard established from a spectrum of the sample containing internal acetone[17]

RESULTS AND DISCUSSION

Intact fetuin. — Fig. 3 shows the ^{13}C-n.m.r. spectrum of native calf fetuin. Partial overlap between resonances of the oligosaccharide and the protein backbone occur only in the regions of C-2 of the 2-acetamido-2-deoxy sugars, C-6 of the hexoses (45–60 p.p.m.), and the non-hydroxylated carbons of α-NeuAc (*e.g.*, C-1, C-3, and C-11)[9,10,13]. The assignments for the various anomeric carbon resonances (inset of Fig. 3) are given in Table I. The detailed assignments are discussed below.

Assuming that all β-D-Gal residues (1→3)-linked to α-D-GalNAc arise from *O*-linked side-chains and that all the other galactosyl residues belong to *N*-linked side-chains, a ratio of ~1:3 was obtained for *O*- and *N*-linked β-D-Gal residues. Since fetuin has an equal number of *O*-linked and *N*-linked chains, the latter, on average, must have a triantennary structure. From considerations of the ^{13}C chemical shifts, one of the three antennas must be either (1→4)- or (1→6)-linked β-D-GlcNAc, whereas the other two are (1→2)-linked β-D-GlcNAc branches, one of which must be linked to α-D-Man-(1→6)[18–20]. The integration ratio between 6- and 3-*O*-substituted (1→4)-linked β-D-Gal residues in the *N*-linked units was ~1:1. Therefore, microheterogeneity is proposed for the distribution of the α-NeuAc linkages.

The non-anomeric (50–85 p.p.m.) region yielded an unusual downfield resonance at 83.4 p.p.m. that could not be assigned to any of the carbohydrate residues discussed so far. One possible assignment was to a glycosylated C-3 of β-D-GlcNAc[21]. Thus, the presence of a small proportion of β-D-Gal (1→3)-linked to β-D-GlcNAc residues further contributes to the heterogeneity of the *N*-linked side-chains.

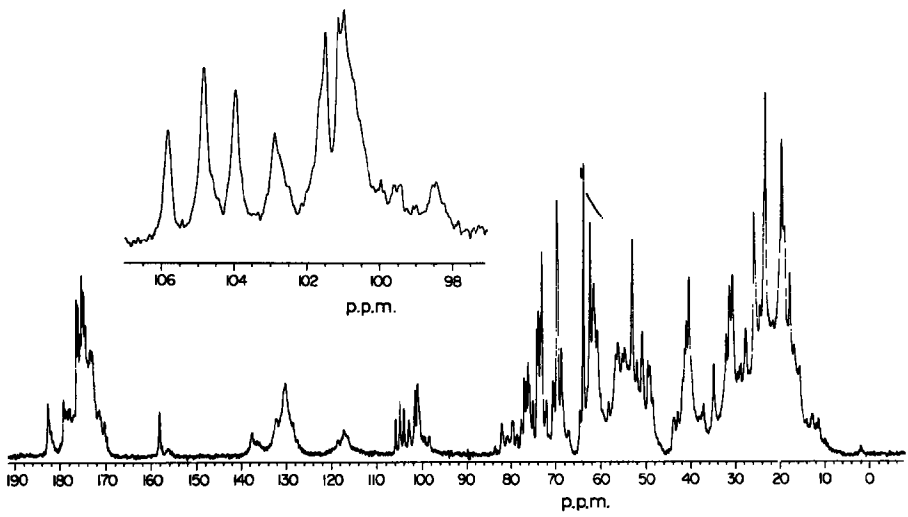


Fig. 3. 60.5-MHz ^{13}C-n.m.r. spectra of fetuin (see Experimental).

TABLE I

^{13}C CHEMICAL SHIFTS (P.P.M.) AND ASSIGNMENTS OF THE ANOMERIC CARBONS OF NATIVE AND ENZYMICALLY TREATED CALF FETUIN[a]

Peak	*Fetuin*			*Assignments*
	Native	*Desialylated*	*Degalactosylated*	
1	105.81	105.98	105.97	β-D-Gal-(1→3)[b]
2	104.85	104.85	104.84	6 β-D-Gal-(1→4), 3 β-D-Gal-(1→3)
3		104.24	104.27	β-D-Gal-(1→4)
4	103.96			3 β-D-Gal-(1→4)
5	102.87	102.83	102.87	4 β-D-GlcNAc-(1→4)
6	[c]	[c]	102.60	β-D-GlcNAc-(1→4)[d]
7	101.66	101.63	101.65	3,6 β-D-Man-(1→4)
8	101.49			α-NeuAc-(2→6)
9	101.14			α-NeuAc-(2→3)
10	100.98			α-NeuAc-(2→3)[e]
11	100.71	100.80	100.96	4 β-D-GlcNAc-(1→2)
12	100.53	100.40	100.49	2,4 α-D-Man-(1→3)
13	99.9		99.8	3 α-D-GalNAc-(1→O)-Thr
14	99.6	99.5	99.5	3 α-D-GalNAc-(1→O)-Ser
15	99.4			3 α-D-GalNAc-(1→O)-Ser
16	98.42	98.41	98.35	2 α-D-Man-(1→6)

[a]See structures **1** and **3**. Numbering preceding the residue designations correspond to the substitution positions on that residue. [b]Bound to α-D-GalNAc. [c]Shoulder to the right of peak 5. [d]Substituted by β-D-Man at C-4. [e]Sialic acid bound to β-D-Gal-(1→3)-α-D-GalNAc.

Most of the prominent and narrow peaks in the spectrum (Fig. 3) can be assigned to the carbons of the more flexible, terminal α-NeuAc residues[16]. Increasing line-widths are observed for the anomeric carbon resonances of β-D-Gal-(1→4) (11 Hz), β-D-GlcNAc-(1→4) (15 Hz), and α-D-Man-(1→6) (40 Hz), reflecting decreasing flexibility for these sugar residues. In the spectrum of denatured intact fetuin (not shown), these resonances are narrower (8, 10, and 23 Hz, respectively), as a direct result of the protein unfolding. After renaturation of the sample, the ^{13}C-n.m.r. spectrum was identical with that of native fetuin, indicating a complete refolding of the protein.

Partially deglycosylated fetuin. — In order to investigate the nature of the structural heterogeneity observed and to clarify other structural features of the side chains, all the NeuAc and most of the galactose were removed enzymically from the protein.

The complete removal of all α-NeuAc was indicated by the disappearance of the resonance corresponding to C-9 (63.85 p.p.m.) from the spectrum. Another unique feature was an upfield shift (2.06 p.p.m.) of most of the resonance intensity at 81.95 p.p.m. This resonance was assigned to C-4 of β-D-GlcNAc substituted by a α-NeuAc-(2→6)-β-D-Gal-(1→ grouping; when the α-NeuAc residue was removed, the resonance was shifted upfield. The same effect was more clearly seen in the spectra of the isolated *N*-linked glycopeptides and also observed for the tri-

saccharide α-NeuAc-(2→6)-β-D-Gal-(1→4)-β-D-GlcNAc[16], for larger oligosaccharides, and for α_1-acid glycoprotein[22]. Such long-range chemical-shift effects must be of conformational origin, and are not likely to be a result of interaction with the bulky polypeptide backbone[16].

Fig. 4 depicts the anomeric regions of the ^{13}C-n.m.r. spectrum of intact fetuin and its desialylated and degalactosylated derivatives, and the chemical shift data and assignments of the resonances are listed in Table I. Peak 1 was unambiguously assigned to β-D-Gal (1→3)-linked to α-D-GalNAc by comparison with the disaccharide unit of antifreeze glycoprotein[11]. After digestion with neuraminidase,

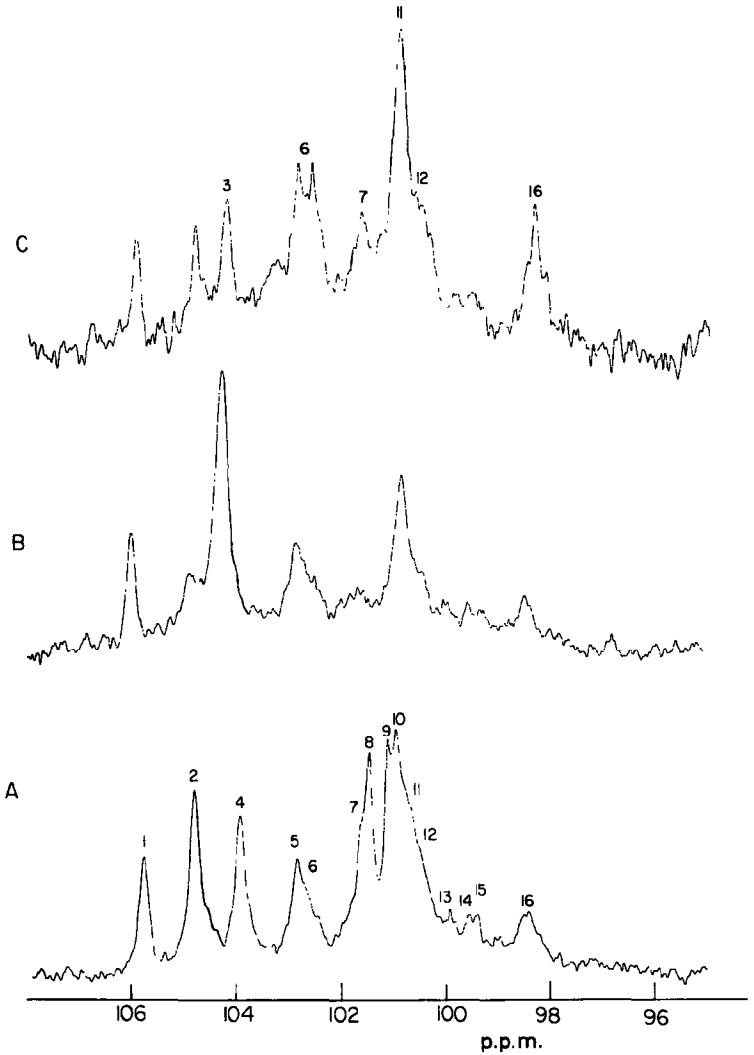


Fig. 4. 60.5-MHz ^{13}C-n.m.r. spectra showing the anomeric carbon region of *A*, fetuin; *B*, neuraminidase-treated fetuin, and *C*, neuraminidase- and β-D-galactosidase-treated fetuin (twice the peak intensity of *A* and *B*) (see Experimental).

peak 1 was shifted downfield to 105.98 p.p.m., similar to the downfield shift (~0.29 p.p.m.) observed for the β-D-Gal-(1→4) residue in model compounds[16]. Peaks 2 and 4 in Fig. 4A must arise[6] from 6- and 3-*O*-α-NeuAc-substituted β-D-Gal-(1→4). The intensity of peak 2, after treatment with the enzymes, remained approximately equal to that of the resonance at ~83.4 p.p.m., which was tentatively assigned to the substituted C-3 of β-D-GlcNAc. Peak 3 corresponds to the non-reducing terminal β-D-Gal that is exposed by the removal of α-NeuAc[16,23]. Peak 5 corresponds to β-D-GlcNAc-(1→4) in the antenna*, and peak 6 to β-D-GlcNAc-(1→4) of the di-*N*-acetylchitobiose unit of the inner core[9,13,18,20]. Peaks 8–10 were assigned to the anomeric carbons (C-2) of α-NeuAc[16] with the aid of the off-resonance decoupled spectrum of intact fetuin. Peak 7, appearing as a shoulder in Fig. 4A, as a broad resonance in Fig. 4B, and a much sharper resonance in Fig. 4C, was unambiguously assigned to β-D-Man[13,18,20]. Peak 11 was assigned to two β-D-GlcNAc-(1→2) residues[9,24,25]. The shoulder, peak 12, was assigned to α-D-Man-(1→3) by comparison with the chemical shifts found for the resonances of that residue in some hybrid glycopeptides isolated from ovalbumin[22]. Peaks 13–15 are assigned to C-1 of α-D-GalNAc linked to serine and threonine[9,11]. Finally, peak 16, the most-upfield anomeric resonance, was assigned to α-D-Man-(1→6) substituted at C-2 by β-D-GlcNAc[9,24,25].

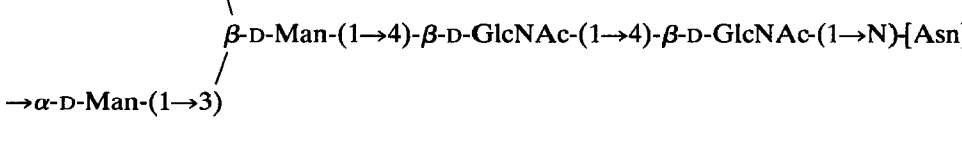
Treatment of the sialic acid-free fetuin with β-D-galactosidase removed 75% of the galactose residues in good agreement with the value reported in the literature[15]. Moreover, it was established that ~85% of the β-D-galactose (1→4)-linked to β-D-GlcNAc was removed compared with an ~65% removal rate for β-D-galactose (1→3)-linked to α-D-GalNAc and no removal of the β-D-Gal (1→3)-linked to β-D-GlcNAc.

N-*linked glycopeptides of calf fetuin.* — Preliminary assignments were based on the assumption that the following sub-structures were present:

α-NeuAc-(2→3)-β-D-Gal-(1→4)-β-D-GlcNAc-(1→,

α-NeuAc-(2→6)-β-D-Gal-(1→4)-β-D-GlcNAc-(1→,

and



*The presence of β-D-GlcNAc-(1→4) rather than β-D-GlcNAc-(1→6) antenna in the structure was inferred, at this stage, from the absence of a resonance in the region 63.2–67.2 p.p.m. which would correspond to the glycosylated C-6 of a β-D-Man residue.

The assignments (Table II) of the resonances corresponding to the first two partial structures followed from those obtained for model oligosaccharides[16]. The chemical shift assignments for the pentasaccharide core unit were made by comparison with similar core units[9,13,18,20]. Additional peak assignments were obtained by calculating the expected chemical shifts for the resonances of C-2 and C-4 of α-D-Man substituted by β-D-GlcNAc residues[19,24–27]. Assignments of still other resonances were consistent with the sub-structure[21,28]

α-NeuAc-(2→3)-β-D-Gal-(1→3)-β-D-GlcNAc→.

Integration of the various resonances, taking into account the differential n.O.e. values and the number of protons attached, yielded three α-NeuAc and three β-D-Gal residues. Similarly, a total of five β-D-GlcNAc residues was determined to be present from the integration of their C-2 resonances. Assuming the presence of only one α-D-Man-(1→6) and one di-*N*-acetylchitobiose unit per *N*-linked side-chain, these results are consistent with a triantennary structure.

In the ^{13}C-n.m.r. spectrum, (2→3)- and (2→6)-linked α-NeuAc residues could be distinguished since their respective carbons are magnetically non-equivalent and thus resonated with slightly different chemical shifts. In the 360-MHz ^{1}H-n.m.r. spectrum of the intact *N*-linked glycopeptide, an additional magnetically different type of α-NeuAc residue was observed, as shown in Fig. 5, which corresponds to the spectral region of the axial H-3 of α-NeuAc[8,23,29,30].

Ion-exchange chromatography can be used for the separation of oligosaccharides and glycopeptides according to the relative number of NeuAc residues and the overall molecular weights[31–36]. Fig. 2 shows the elution profile for DEAE Sephadex chromatography of the fetuin-derived, *N*-linked glycopeptides. Analysis of the ^{1}H- and the ^{13}C-n.m.r. spectra of the isolated fractions showed that fraction *A5* carried all of the resonance intensity at 83.43 p.p.m. and that the resonance at 104.88 p.p.m. was twice as large as that at 103.94 p.p.m. Equal amounts of the three magnetically non-equivalent α-NeuAc residues were observed, as discussed above, in the ^{1}H-n.m.r. spectrum of fraction *A5* (not shown). Thus, the ion-exchange chromatography yielded a unique fraction (fraction *A5*) which contained one α-D-Gal-(1→3) residue per structure. This fraction included one α-NeuAc-(2→6) and two α-NeuAc-(2→3) residues, with one of the latter linked to β-D-Gal-(1→3).

As with the intact glycoprotein, complete digestion of the sialic acid was also observed with the glycopeptide fraction. The anomeric region of the spectrum integrated to ten carbohydrate residues in accordance with the proposed triantennary structure. At this stage, it was possible to state categorically that fraction *A* must be homogeneous with respect to the type of branch point. With a mixture of two triantennary structures, in which the β-D-GlcNAc-(1→4) antenna is linked to either of the α-D-Man residues (see structures **1** and **2**), a "doubling" of the resonances corresponding to the α-D-Man residues as well as those for β-D-GlcNAc-(1→4) would have been observed. Therefore, only one of the previously proposed triantennary structures is correct.

TABLE II

CHEMICAL SHIFTS (±0.05 P.P.M.) AND ASSIGNMENTS FOR THE *N*-LINKED GLYCOPEPTIDES OF FETUIN[a]

Carbon atom	*Fetuin*		
	Native	*Desialylated*	*Degalactosylated*
β-D-GlcNAc-(1→N)			
1	79.45	79.48	79.48
2	55.01	55.00	55.09
3	74.16	74.14	74.10
4	80.88&80.87	80.72	80.90&80.80
5	77.50	77.50	77.52
6	61.55/61.30	61.29	61.30/61.39
β-D-GlcNAc-(1→4)[b]			
1	102.56	102.58	102.55
2	56.48	56.48	56.32
3	73.36	73.31/73.38	73.30
4	80.09	80.00	80.19
5	75.73	75.70	75.74
6	61.30/61.55	61.29	61.39/61.30
β-D-Man-(1→4)			
1	101.63	101.67	101.63
2	71.45	71.44	71.44
3	81.74	81.76	81.74
4	67.28–67.03	66.98	67.27–67.11
5	75.73	75.70	75.74
6	67.03–67.28	66.98	67.11–67.27
α-D-Man-(1→3)			
1	100.41	100.42	100.44, 103.24[c]
2	77.76/77.50	77.70/77.49	77.88/77.37, 69.96[c]
3	73.06	72.90	72.96, 71.77[d]
4	79.45	79.48	79.48, 68.16[d], 79.05[c]
5	73.82	73.83	73.90, 75.52[d]
6	63.37	73.37	63.45, 62.92[d]
α-D-Man-(1→6)			
1	98.42	98.48	98.42, 100.95[c]
2	77.50/77.76	77.32/77.70	77.37/77.88, 69.45[c]/62.62[c]
3	70.76	70.75	70.80/70.87, 71.10[c]
4	68.69/68.83	68.56	68.73
5	74.16	74.14	74.20
6	62.55/62.93	62.52/62.93	62.55/62.92

β-D-GlcNAc-(1→2,4)			
1	102.83, 100.82[e]	102.84, 100.79[e]	102.87&102.79, 101,01[e]
2	56.27–56.00	56.24&56.11, 55.92[f]	56.97&56.71, 55.95[f]
3	73.36, 83.40[f]	73.38, 83.71[f]&83.55[f]	74.82–74.66, 83.71[f]&83.30[f]
4	81.87[g], 79.75[h], 69.74[f]	79.89	71.28–71.10, 69.96[f]
5	75.73[g], 76.05[h]	76.00, 76.29[f]	77.23&77.16
6	61.30[h], 61.55[g], 61.96[f]	61.29, 61.98[f]	62.29&62.08, 61.99[f]
β-D-Gal-(1→4)			
1	104.79[i], 103.96[j]	104.24	104.20
2	72.08[i], 70.68[j]	72.27	72.31
3	73.82[i], 76.43[j]	73.83	73.90
4	69.48[i], 68.83[j]/68.69[j]	69.85	69.88
5	74.97[i], 76.78[j]	76.64	76.75
6	64.62[i], 62.29[j]	62.30	62.29
β-D-Gal-(1→3)			
1	104.79	104.81	104.82
2	70.40	71.95	72.01
3	(−)	73.84	73.90
4	(−)	69.43	69.45&69.62
5	(−)	(−)	(75.52)
6	(−)	(−)	62.29
α-NeuAc-(2→X)			
1	174.73[k], 175.08[l]		
2	101.52[k], 101.17[l]		
3	41.35[k], 40.95[l]		
4	69.74/69.59/69.48		
5	53.22[k], 53.04[l]		
6	73.82[k], 74.16[l]		
7	69.74/69.59/69.48		
8	73.06		
9	64.00		
10	176.29[k], 176.21[l]		

[a]Spectral conditions are given in the Experimental section. All the signals corresponding to the $COCH_3$ groups are found in the region 23.83–23.38 p.p.m. The $COCH_3$ signals of β-D-GlcNAc residues are found in the region 176.01–175.85 p.p.m. The signs used in the table are: &, both signals are to be assigned to the same carbon due to presence of more than one possible isomer: /, assignment may be interchanged with another carbon; –, the signal multiplet covered by this range is assigned to the particular carbon; a comma separating two chemical shifts indicates different sugar residues as defined by each corresponding superscript; (−), chemical shift value was not determined. [b]Linked to a β-D-GlcNAc-(1→N) residue. [c]Mannose residue for which the substituting β-D-GlcNAc residue at position 2 was removed by enzymic degration. [d]Mannose residue for which the substituting β-D-GlcNAc residue at position 4 was removed by enzymic degration. [e]β-D-GlcNAc-(1→2) residue. [f]GlcNAc substituted at position 3 by a β-D-Gal residue. [g]GlcNAc substituted at position 4 by an α-NeuAc-(2→6)-β-D-Gal unit. [h]GlcNAc substituted at position 4 by an α-NeuAc-(2→3)-β-D-Gal unit. [i]Galactose substituted at position 6 by an α-NeuAc residue. [j]Galactose substituted at position 3 by an α-NeuAc residue. [k] X = 6. [l]X = 3.

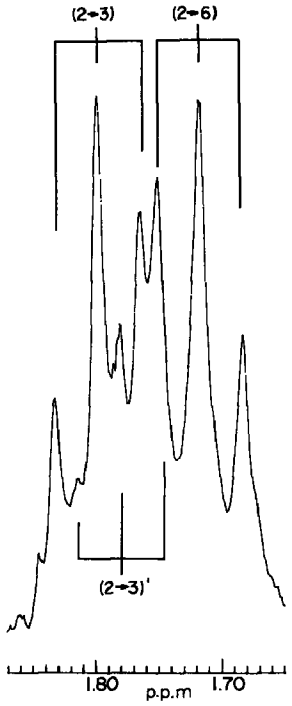


Fig. 5. A 360-MHz ^{1}H-n.m.r. spectrum of the intact *N*-linked glycopeptide from fetuin, showing the spectral region corresponding to the H-3*a* protons of the α-NeuAc residues; 8mM solution in "100%" D_2O, pH ~6.0, and 40 scans (for other details, see Experimental).

A minor component that carries β-D-Gal-(1→3) was also present in the mixture and it comprised 12% of the total galactose content. Its presence in the spectrum was indicated by the anomeric resonance at 104.81 p.p.m. and by additional peaks in the non-anomeric region of the spectrum (Table II)[21].

Fig. 6 shows the ^{13}C-n.m.r. spectrum obtained following digestion of the desialylated fraction A with β-D-galactosidase. From n.m.r. integration results, it was concluded that nearly 85% of the total galactose was removed, leaving essentially only the β-D-Gal-(1→3) residues. Additional resonances at 103.24, 102.87, 102.79, and 100.95 p.p.m. arose as a result of the action of β-*N*-acetylglucosaminidase present in small quantities in the commercial β-D-galactosidase preparation (see Experimental), which resulted in the digestion of about one β-D-GlcNAc-(1→2) residue per *N*-linked side-chain.

The peak at 103.24 p.p.m. was assigned to an α-D-Man-(1→3) from which a β-D-GlcNAc-(1→2) residue was removed. Therefore, the combined resonance intensities of the peaks at 103.24 and 100.44 p.p.m. should correspond to one carbon, which is indeed the case. Similarly, the peak at 100.95 p.p.m. was assigned to a terminal α-D-Man-(1→6) residue and it integrates together with the peak at 98.42 p.p.m. to one sugar residue. The peak at 102.87 p.p.m. corresponds to the substructure β-D-Gal-(1→3)-β-D-GlcNAc-(1→4)-, and it has one-half the intensity of

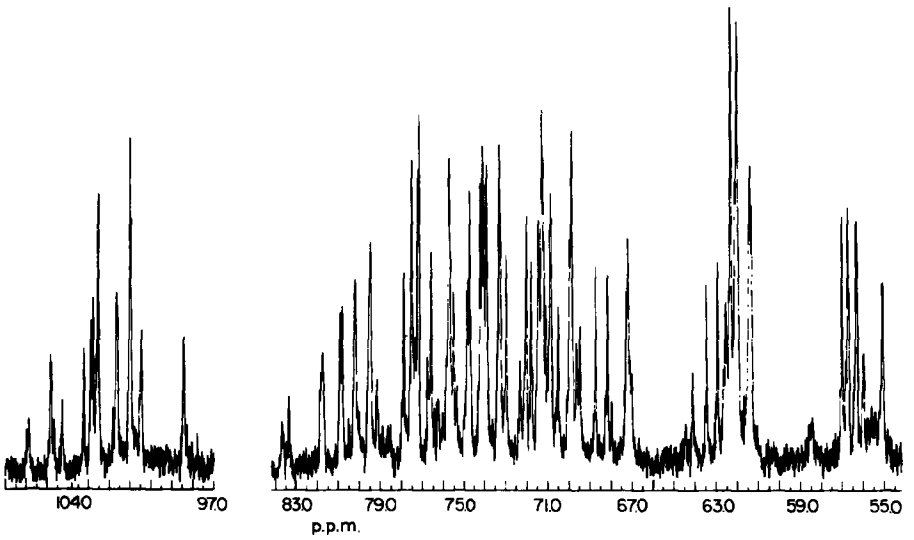


Fig. 6. A 90.56-MHz ^{13}C-n.m.r. spectrum of β-D-galactosidase-treated, sialic acid-free, *N*-linked glycopeptides from fetuin; 20 mg/mL solution, pH 8.2 in a 5-mm n.m.r. tube, 17,200-Hz spectral window, 60° pulse, 1-s recycle time, 40,000 scans, 32k data points, and 1.5-Hz line-broadening.

the resonance at 102.79 p.p.m. which corresponds to the sub-structure β-D-Gal-(1→4)-β-D-GlcNAc-(1→4)-.

The assignments and the integration results given above are supported by additional evidence in the non-anomeric spectral region. Thus, the spectral region corresponding to the C-2 resonances of hexosamines[9,10] revealed that the β-D-GlcNAc residue that was enzymically removed was present mainly in a (1→2) linkage.

From chemical shift considerations, it is proposed that the branching pattern of structure **1** is the correct triantennary structure for the *N*-linked carbohydrate units of calf fetuin. With the branching pattern of structure **2**, the removal of a β-D-GlcNAc-(1→2) residue from α-D-Man-(1→3) should give rise to a resonance in the 103.7–103.5 p.p.m. region[13,18–20]. Clearly, there is no discernible resonance in that region. The removal of β-D-GlcNAc-(1→2) linked to α-D-Man-(1→6) in structure **1** should yield a resonance around 101 p.p.m.[13,18–22], and indeed such a signal was observed at 100.95 p.p.m. Also, substitution of a terminal α-D-Man by β-D-GlcNAc-(1→4) should lead to an ~0.5 p.p.m. upfield shift[18,22] relative to the position for a non-reducing terminal α-D-Man-(1→3), and such a resonance is found. On the other hand, if a β-D-GlcNAc-(1→4) residue were linked to α-D-Man-(1→6) (structure **2**), we would expect an additional resonance in the vicinity of 100.44 p.p.m. This resonance is clearly not observed.

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