Kastrup, R. V., & Schmidt, P. G. (1978) *Nucleic Acids Res. 5*, 257–269.
Kim, S.-H. (1979) in *Transfer RNA: Structure, Properties, and Recognition* (Schimmel, P. R., Soll, D., & Abelson, J. N., Eds.) pp 83–100, Cold Spring Harbor Laboratories, Cold Spring Harbor, NY.
Kopper, R. A., Schmidt, P. G., & Agris, P. F. (1983) *Biochemistry 22*, 1396–1401.
Lowrie, R. J., & Bergquist, P. L. (1968) *Biochemistry 7*, 1761–1770.
Massoulie, J., Michelson, A. M., & Pochon, F. (1963) *Biochim. Biophys. Acta 114*, 16–26.
Nielsen, P. E., & Leick, V. (1985) *Eur. J. Biochem. 152*, 619–623.
O'Connor, T. P., & Coleman, J. E. (1982) *Biochemistry 21*, 848–854.
Ofengand, J., Bierbaum, J., Horowitz, J., Ou, C.-N., & Ishaq. M. (1974) *J. Mol. Biol. 88*, 313–325.
Olsen, J. I., Schweizer, M. P., Walkiw, I. J., Hamill, W. D., Horton, W. J., & Grant, D. M. (1982) *Nucleic Acids Res. 10*, 4449–4464.
Ou, C.-N., & Song, P.-S. (1978) *Biochemistry 17*, 1054–1059.
Ramberg, E. S., Ishaq, M., Rulf, S., Moeller, B., & Horowitz, J. (1978) *Biochemistry 17*, 3978–3985.
Randerath, K., Randerath, E., Chia, L., & Nowak, B. J. (1974) *Anal. Biochem. 59*, 263–271.
Reid, B. R. (1981) *Annu. Rev. Biochem. 50*, 969–996.
Reid, B. R., & Hare, D. R. (1982) in *Mobility and Function in Proteins and Nucleic Acids* (CIBA Foundation Symposium 93) pp 208–225, Pittman, London.
Rich, A., & RajBhandary, U. L. (1976) *Annu. Rev. Biochem. 45*, 806–860.
Robertson, D. E., Kroon, P. A., & Ho, C. (1977) *Biochemistry 16*, 1443–1451.
Roy, S., & Redfield, A. G. (1983) *Biochemistry 22*, 1386–1390.
Sander, E. G., & Deyrup, C. L. (1972) *Arch. Biochem. Biophys. 150*, 600–605.
Scheit, K. H., & Gaertner, K. G. (1969) *Biochim. Biophys. Acta 182*, 1–9.
Schulman, L. H., & Pelka, H. (1977) *J. Biol. Chem. 252*, 814–819.
Shapiro, R., Servis, R. E., & Welcher, M. (1970) *J. Am. Chem. Soc. 92*, 422–424.
Shaw, D. (1976) in *Fourier Transform NMR Spectroscopy*, p 179, Elsevier, New York.
Sigler, P. B. (1975) *Annu. Rev. Biophys. Bioeng. 4*, 477–527.
Sprinzl, M., Moll, J., Meissner, F., & Hartmann, T. (1985) *Nucleic Acids Res. 13*, r1–r49.
Steinmetz-Kayne, M., Benigno, R., & Kallenbach, N. R. (1977) *Biochemistry 16*, 2064–2073.
Sykes, B. D., & Hull, W. E. (1978) *Methods Enzymol. 49*, 270–295.
Sykes, B. D., & Weiner, J. H. (1980) in *Magnetic Resonance in Biology* (Cohen, J. S., Ed.) Vol. 1, pp 171–196, Wiley, New York.
Szer, W., & Shugar, D. (1963) *Acta Biochim. Pol. 10*, 219–231.
Thompson, J. F., Bachellerie, J.-P., Hall, K., & Hearst, J. E. (1982) *Biochemistry 21*, 1363–1368.
Tropp, J., & Redfield, A. G. (1981) *Biochemistry 20*, 2133–2140.
Vlassov, V. V., Giege, R., & Ebel, J.-P. (1981) *Eur. J. Biochem. 119*, 51–59.
Wempen, I., Duschinsky, L., Kaplan, L., & Fox, J. J. (1961) *J. Am. Chem. Soc. 83*, 4755–4766.
Yaniv, M., & Barrell, B. G. (1969) *Nature (London) 222*, 278–279.

Asparagine-Linked Sugar Chains of Fetuin: Occurrence of Tetrasialyl Triantennary Sugar Chains Containing the Galβ1→3GlcNAc Sequence†

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ABSTRACT: Asparagine-linked sugar chains were quantitatively released from fetuin by hydrazinolysis. Structural analysis of the sugar chains by sequential exoglycosidase digestion in combination with methylation analysis and Smith degradation revealed that most of them have typical biantennary (8%) and triantennary (74%) structures containing different amounts of *N*-acetylneuraminic acid residues. In addition, an unusual tetrasialyl triantennnary sugar chain (17%) containing the Galβ1→3GlcNAc sequence in the outer chain moiety was detected, and its structure was elucidated as NeuAcα2→3Galβ1→3(NeuAcα2→6)-GlcNAcβ1→4(NeuAcα2→6Galβ1→4GlcNAcβ1→2)Manα1→3(NeuAcα2→3Galβ1→4GlcNAcβ1→-2Manα1→6)Manβ1→4GlcNAcβ1→4GlcNAc.

Fetuin, one of the major glycoproteins found in fetal calf serum, has been shown to contain three mucin-type sugar chains (Spiro & Bhoyroo, 1974) and three asparagine-linked sugar chains in one molecule (Spiro, 1973). Both types of sugar chains have been extensively investigated; however, the structures of asparagine-linked sugar chains reported by Baenziger and Fiete (1979) and by Nilsson et al. (1979) were different. The sugar chains proposed by the two groups were the same with respect to having a trisialyl triantennary structure with 2,4-branched outer chains but were opposite in regard to the location of the outer chain branch. Later, Krusius and Finne (1981) presented the data that the 2,4 branch was located on the Manα1→3 side in accordance with the result obtained by Nilsson et al. (1979). While studying

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the structures of the asparagine-linked sugar chains of the human promyelocytic leukemia cell line, HL-60, we found that a tetrasialyl asparagine-linked sugar chain, which was absent in undifferentiated cells, was included in differentiated cells (reported at the 12th International Congress of Biochemistry, Perth, Western Australia, 1982). Further studies of this interesting phenomenon revealed that the sugar chain is not included in the plasma membrane glycoproteins of the cells, but in the fetuin absorbed on the surface of differentiated cells from culture medium. Since tetrasialyl asparagine-linked sugar chain has never been reported to occur in fetuin, we reinvestigated the whole structures of the asparagine-linked sugar chains of the glycoprotein. The results reported in this paper indicated that the asparagine-linked sugar chains of fetuin are much more complex than they have been considered.

Materials and Methods

Chemicals and Enzymes. NaB^3H_4 (360 mCi/mmol) was purchased from New England Nuclear, Boston, MA. NaB^2H_4 and $NaIO_4$ were obtained from Merck Co., Darmstadt. Fetuin was obtained from Sigma Chemical Co., St. Louis, MO, or was purified in our laboratory from fetal calf serum (Irvine Scientific, Santa Ana, CA) by the method of Spiro (1960). β-Galactosidase, β-*N*-acetylhexosaminidase, and α-mannosidase were purified from jack bean meal according to the method of Li and Li (1972). Diplococcal β-galactosidase and β-*N*-acetylhexosaminidase were prepared as previously described (Kobata & Takasaki, 1978). Sialidase from *Arthrobacter ureafaciens* (Uchida et al., 1974) was purchased from Nakarai Chemicals, Ltd., Kyoto.

Glycosidase digestion was carried out as previously described (Yoshima et al., 1980), unless otherwise noted.

Release of Asparagine-Linked Sugar Chains of Fetuin. The purified fetuin (34 mg) was subjected to hydrazinolysis as previously described (Takasaki et al., 1982). One-fourth of the oligosaccharide fraction was reduced with NaB^3H_4, and 7.9×10^6 cpm of the radioactivity was incorporated. By comparing the radioactivity with that incorporated into xylose used as an internal standard sugar, the amount of sugar chains released from 1 mol of fetuin (4.8×10^4 daltons) was calculated to be 2.4 mol. The remaining fraction was reduced with NaB^2H_4 for methylation analysis. One-third of the radioactive oligosaccharide fraction was added to the NaB^2H_4-reduced sample for easy detection of oligosaccharides during the separation procedures.

Preparation of Sialic Acid Labeled Fetuin and Its Oligosaccharides. The purified fetuin was labeled according to the method of Lenten and Ashwell (1972) as follows. Fetuin (2.2 mg) was oxidized with 1 mL of 4.75 mM $NaIO_4$ in 0.1 M acetate buffer, pH 5.5, at 0 °C in the dark for 10 min. After addition of 20 μL of ethylene glycol followed by 1 h of incubation, the product was dialyzed against distilled water and then lyophilized. The product was subsequently reduced with 0.4 mCi of NaB^3H_4 in 300 μL of 0.05 M borate buffer, pH 9.5, at 30 °C for 3 h, followed by 30 min of incubation after addition of 2 mg of $NaBH_4$. The reaction mixture was acidified by adding acetic acid and dialyzed against distilled water. Total radioactivity of the fetuin preparation thus obtained was 1.4×10^7 cpm. A part of the labeled fetuin (55 μg, 3.5×10^5 cpm) was subjected to SDS–PAGE.[1] The gel was cut into 5-mm slices and was extracted with 1 mL of 0.1% SDS by continuous shaking for 1 day at room temperature. Aliquots were withdrawn for determination of radioactivity. The eluted sample corresponding to fetuin was dialyzed against distilled water, lyophilized, and dried in vacuo over P_2O_5. Its oligosaccharide pattern was examined by hydrazinolysis as described above, except that reduction was performed by using $NaBH_4$.

Oligosaccharides. NeuAcα2→6Galβ1→4GlcNAcβ1→2Manα1→6(NeuAcα2→6Galβ1→4GlcNAcβ1→2Manα1→3)Manβ1→4GlcNAcβ1→4GlcNAc$_{OT}$[2] ($NeuAc_2Gal_2GlcNAc_2Man_3GlcNAcGlcNAc_{OT}$) and NeuAcα2→6Galβ1→4GlcNAcβ1→2Manα1→6(or 3)[Galβ1→4GlcNAcβ1→2Manα1→3(or 6)]Manβ1→4GlcNAcβ1→4GlcNAc$_{OT}$ ($NeuAcGal_2GlcNAc_2Man_3GlcNAcGlcNAc_{OT}$) were prepared from human transferrin (Spik et al., 1975) by hydrazinolysis. NeuAcα2→6Galβ1→4GlcNAcβ1→2(NeuAcα2→3Galβ1→4GlcNAcβ1→4)Manα1→3(NeuAcα2→6Galβ1→4GlcNAcβ1→2Manα1→6)Manβ1→4GlcNAcβ1→4GlcNAc$_{OT}$ ($NeuAc_3Gal_3GlcNAc_3Man_3GlcNAcGlcNAc_{OT}$) was from human ceruloplasmin, and its mono- and disialyl oligosaccharides ($NeuAc_1$- and $NeuAc_2Gal_3GlcNAc_3Man_3GlcNAcGlcNAc_{OT}$) were prepared by mild acid hydrolysis of $NeuAc_3Gal_3GlcNAc_3Man_3GlcNAcGlcNAc_{OT}$ (Yamashita et al., 1981a). Galβ1→4GlcNAcβ1→4(Galβ1→4GlcNAcβ1→2)Manα1→3(Galβ1→4GlcNAcβ1→2Manα1→6)Manβ1→4GlcNAcβ1→4GlcNAc$_{OT}$ ($Gal_3GlcNAc_3Man_3GlcNAcGlcNAc_{OT}$) and Galβ1→4GlcNAcβ1→2Manα1→6(Galβ1→4GlcNAcβ1→2Manα1→3)Manβ1→4GlcNAcβ1→4GlcNAc$_{OT}$ ($Gal_2GlcNAc_2Man_3GlcNAcGlcNAc_{OT}$) were obtained by sialidase digestion of $NeuAc_3Gal_3GlcNAc_3Man_3GlcNAcGlcNAc_{OT}$ and $NeuAc_2Gal_2GlcNAc_2Man_3GlcNAcGlcNAc_{OT}$, respectively. Manβ1→4GlcNAcβ1→4XylNAc$_{OT}$ ($ManGlcNAcXylNAc_{OT}$) and GlcNAcβ1→2(GlcNAcβ1→4)Manα1→3Manβ1→4GlcNAcβ1→4XylNAc$_{OT}$ ($GlcNAc_2Man_2GlcNAcXylNAc_{OT}$) were prepared from asialooligosaccharides of transferrin and ceruloplasmin, respectively, by Smith degradation. Other standard oligosaccharides were obtained by exoglycosidase digestion of the oligosaccharides listed above.

Analytical Methods. High-voltage paper electrophoresis was carried out at 80 V/cm for 4 h by using pyridine/acetate buffer, pH 5.4 (pyridine/acetic acid/water, 3:1:387). Descending paper chromatography was performed by using solvent I (1-butanol/ethanol/water, 4:1:1), solvent II (ethyl acetate/pyridine/acetic acid/water, 5:5:1:3), or solvent III (1-butanol/acetic acid/water, 12:3:5). Bio-Gel P-4 column chromatography was performed as previously described (Yamashita et al., 1982). Methylation analysis was carried out as described by Endo et al. (1979). Smith degradation and identification of sialic acid were performed according to the previously described method (Takasaki et al., 1984). SDS–PAGE was carried out by using 10% gel according to the method of Laemmli (1970). Proteins and sugars were visualized by staining the gel with Coomassie brilliant blue and periodic acid–Schiff's reagent, respectively (Fairbanks et al., 1971).

Results

Fractionation of Oligosaccharides Released from Fetuin by Hydrazinolysis. When an oligosaccharide mixture prepared by hydrazinolysis of fetuin followed by NaB^3H_4 reduction was subjected to paper electrophoresis at pH 5.4, 99% of the total oligosaccharides showed an acidic nature and were separated

[1] Abbreviations: SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; NeuAc, *N*-acetylneuraminic acid; Gal, galactose; GlcNAc, *N*-acetylglucosamine; Man, mannose; XylNAc, *N*-acetylxylosamine.

[2] The subscript OT is used to indicate NaB^3H_4-reduced oligosaccharides. All sugars mentioned in this paper are of the D configuration except for fucose, which has the L configuration.

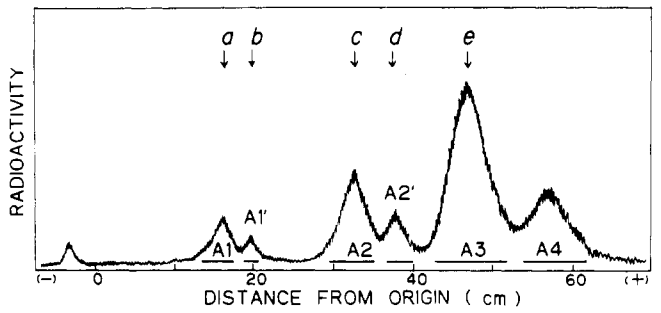


FIGURE 1: Paper electrophoresis at pH 5.4 of oligosaccharides released from fetuin by hydrazinolysis. Arrows indicate positions of standard oligosaccharides: (a, c, and e) $NeuAc_1$-, $NeuAc_2$-, and $NeuAc_3Gal_3GlcNAc_3Man_3GlcNAcGlcNAc_{OT}$, respectively; (b and d) $NeuAc_1$- and $NeuAc_2Gal_2GlcNAc_2Man_3GlcNAcGlcNAc_{OT}$, respectively.

into six fractions with molar ratios of 6% (A-1), 2% (A-1′), 25% (A-2), 6% (A-2′), 43% (A-3), and 17% (A-4), respectively (Figure 1). A-1, A-2, and A-3 had the same electrophoretic mobility as authentic mono-, di-, and trisialyl triantennary oligosaccharides, respectively, and A-1′ and A-2′ had the same mobility as mono- and disialyl standard oligosaccharides with biantennary structure. A-4 migrated faster than trisialyl triantennary oligosaccharides, suggesting that this oligosaccharide is a component that has never been identified in the previous studies (Nilsson et al., 1979; Baenziger & Fiete, 1979; Krusius & Finne, 1981). Partial removal of sialic acid residues from A-4 by heating in 0.01 N HCl at 100 °C for 3 min produced three additional slower moving acidic components and a neutral component (data not shown), indicating that this new component is a tetrasialyl oligosaccharide.

All acidic components in A-1 to A-4 were completely converted to neutral ones by sialidase digestion, and the products were named as A-1N to A-4N, respectively. The sialic acid residues directly released from fetuin by sialidase digestion were reduced with NaB^3H_4. Analysis of the radioactive sialic acid alcohol by paper chromatography using solvent III revealed that only *N*-acetylneuraminic acid was found as a radioactive component. When subjected to Bio-Gel P-4 column chromatography, A-1N, A-2N, and A-3N were eluted as a single peak at the position of 16.2 glucose units (Figure 2A,C,E). A-4N gave a single peak with 15.8 glucose units (Figure 2F). A-1′N and A-2′N were eluted at the position of a typical biantennary sugar chain (Figure 2B,D).

Structural Analysis of A-4. When A-4N was digested with jack bean β-galactosidase, three galactose residues were released (Figure 3A). Subsequent digestion of the product with diplococcal and then jack bean β-*N*-acetylhexosaminidases converted it to radioactive trimannosyl core oligosaccharide, $Man_3GlcNAcGlcNAc_{OT}$, releasing two *N*-acetylglucosamine residues (Figure 3B) and one *N*-acetylglucosamine residue (Figure 3C), respectively. These results indicated that A-4N is a triantennary sugar chain. Since diplococcal β-*N*-acetylhexosaminidase hydrolyzes only β1→2 linkages at nonbranched points or in the GlcNAcβ1→2(GlcNAcβ1→4)Man group but not in the GlcNAcβ1→2(GlcNAcβ1→6)Man group (Yamashita et al., 1981b), it was also suggested from the results described above that A-4N contains 2,4-branched outer chains linked to one of the two α-mannosyl residues in the core oligosaccharide.

When A-4N was digested with a mixture of diplococcal β-galactosidase and β-*N*-acetylhexoaminidase, a radioactive product with a size of 10.0 glucose units was obtained (Figure 3D), indicating that one of the three Gal→GlcNAc outer chains remained intact. Further digestion of this radioactive

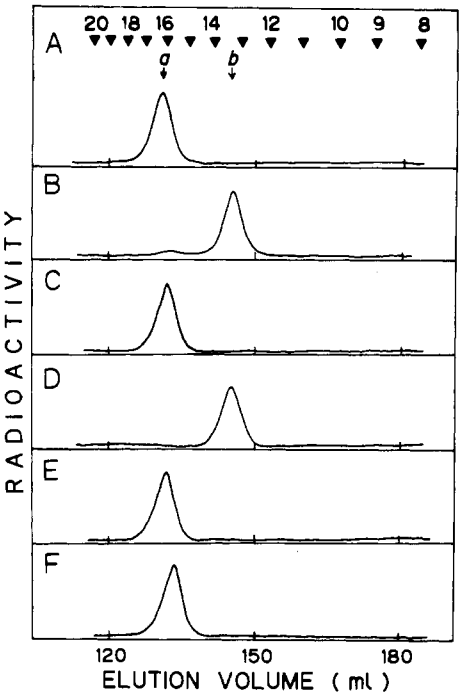


FIGURE 2: Bio-Gel P-4 column chromatography of asialooligosaccharides: (A) A-1N; (B) A-1′N; (C) A-2N; (D) A-2′N; (E) A-3N; (F) A-4N. Black triangles indicate elution positions of glucose oligomers, and numbers indicate glucose units. Arrows a and b are elution positions of authentic $Gal_3GlcNAc_3Man_3GlcNAcGlcNAc_{OT}$ and $Gal_2GlcNAc_2Man_3GlcNAcGlcNAc_{OT}$, respectively.

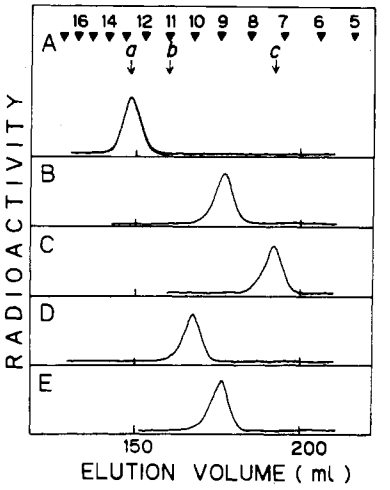


FIGURE 3: Sequential exoglycosidase digestion of A-4N. Exoglycosidase digests were analyzed by Bio-Gel P-4 column chromatography: (A) A-4N digested with jack bean β-galactosidase (1.5 units); (B) the peak in (A) digested with diplococcal β-*N*-acetylhexosaminidase; (C) the peak in (B) digested with jack bean β-*N*-acetylhexosaminidase; (D) A-4N digested with a mixture of diplococcal β-galactosidase and β-*N*-acetylhexosaminidase; (E) the peak in (D) digested with jack bean β-galactosidase (1.5 units). Black traingles are the same as in Figure 2. Arrows a, b, and c indicate elution positions of authentic $GlcNAc_3Man_3GlcNAcGlcNAc_{OT}$, $GlcNAc_2Man_3GlcNAcGlcNAc_{OT}$ and $Man_3GlcNAcGlcNAc_{OT}$, respectively.

product with jack bean β-galactosidase resulted in the release of a galactosyl residue (Figure 3E). This galactosyl linkage was supposed to be either β1→3 or β1→6 but not β1→4 from the aglycon specificity of diplococcal β-galactosidase (Paulson et al., 1978), and it was finally identified as β1→3 by the detection of 4,6-di-*O*-methyl-2-(*N*-methylacetamido)-2-deoxyglucitol in the methylation study (Table I). Its location was also determined to be on the GlcNAcβ1→4Man side, since the degalactosylated product in Figure 3E was converted to

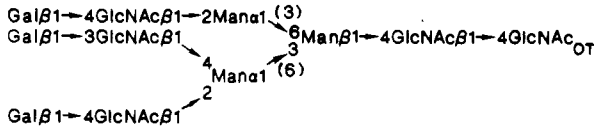
Table I: Methylation Analysis of A-3, A-4, and Their Asialooligosaccharides

methylated sugar	molar ratio[a] A-3	A-3N	A-4	A-4N
galactitol				
2,3,4,6-tetra-*O*-methyl (1,5-di-*O*-acetyl)	–[b]	3.4	–	3.3
2,4,6-tri-*O*-methyl (1,3,5-tri-*O*-acetyl)	1.8	–	1.9	–
2,3,4-tri-*O*-methyl (1,5,6-tri-*O*-acetyl)	1.2	–	1.2	–
mannitol				
3,4,6-tri-*O*-methyl (1,2,5-tri-*O*-acetyl)	1.2	1.3	1.1	1.2
3,6-di-*O*-methyl (1,2,4,5-tetra-*O*-acetyl)	1.1	1.2	1.1	0.9
2,4-di-*O*-methyl (1,3,5,6-tetra-*O*-acetyl)	1.0	1.0	1.0	1.0
2-(*N*-methylacetamido)-2-deoxyglucitol				
1,3,5,6-tetra-*O*-methyl (4-mono-*O*-acetyl)	0.7	0.6	0.7	0.7
3,6-di-*O*-methyl (1,4,5-tri-*O*-acetyl)	3.5	3.6	2.7	2.6
4,6-di-*O*-methyl (1,3,5-tri-*O*-acetyl)	–	–	–	0.8
4-mono-*O*-methyl (1,3,5,6-tetra-*O*-acetyl)	–	–	0.6	–

[a] Numbers were calculated by setting the value for 2,4-di-*O*-methylmannitol as 1.0. [b] Not detected or less than 0.1.

the trimannosyl core oligosaccharide, releaseing an *N*-acetylglucosaminyl residue by jack bean β-*N*-acetylhexosaminidase digestion, but not by diplococcal β-*N*-acetylhexosaminidase treatment (not shown).

From the data thus far described, the following structure is proposed for A-4N:



In order to determine the location of the 2,4 branch, two cycles of Smith degradation were performed on the radioactive A-4N. By the first cycle of degradation, a radioactive hexasaccharide with 10.2 glucose units was produced. This product corresponds to GlcNAcβ1→2(GlcNAcβ1→4)Manα1→3(or 6)-Manβ1→4GlcNAcβ1→4XylNAc$_{OT}$ (Figure 4A). When the product was then subjected to the second cycle of degradation, only one radioactive tetrasaccharide with a mobility of 6.3 glucose units was obtained (Figure 4B). The mobility accords to that of the authentic tetrasaccharide Manα1→3Manβ1→-4GlcNAcβ1→4XylNAc$_{OT}$. If the 2,4-branched outer chains are located on the Manα1→6 side, a radioactive disaccharide, GlcNAcβ1→4XylNAc$_{OT}$, with 4.3 glucose units should be obtained. Therefore, it was concluded that the 2,4-branched outer chains are exclusively located on the Manα1→3 side.

In order to determine the locations of sialic acid residues, A-4 and A-4N fractions were subjected to a methylation study. As shown in Table I, two residues of 2,4,6-tri-*O*-methylgalactitol and one residue each of 2,3,4-tri-*O*-methylgalactitol and 4-mono-*O*-methyl-2-(*N*-methylacetamido)-2-deoxyglucitol were detected from A-4 but not from A-4N. This result indicated that sialic acid linkages occur as the NeuAcα2→-3Gal, the NeuAcα2→6Gal, and the NeuAcα2→6(Galβ1→-3)GlcNAc linkages in a ratio of 2:1:1. In order to determine the location of the NeuAcα2→3Gal linkage in A-4, radioactive A-4 was subjected to Smith degradation. Analysis of the reaction mixture by Bio-Gel P-4 column chromatography revealed that a single radioactive component with mobility of

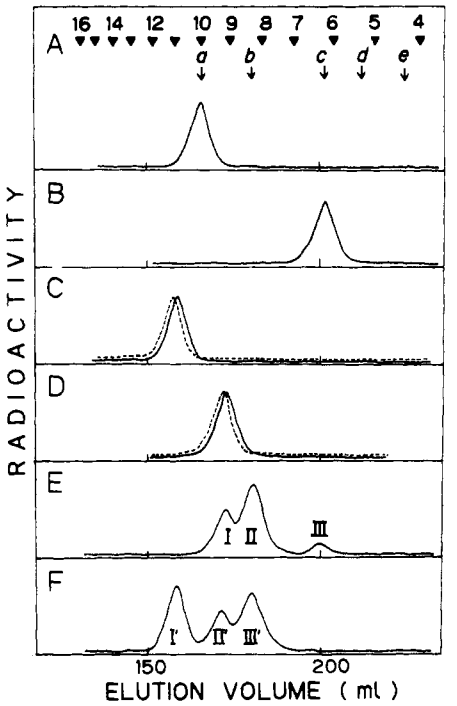


FIGURE 4: Location of branches and *N*-acetylneuraminic acid residues. Smith degradation products and their exoglycosidase digests were analyzed by Bio-Gel P-4 column chromatography: (A) Smith degradation product of A-4N; (B) the product of A-4N subjected to two cycles of Smith degradation; (C) Smith degradation products of A-4 (solid line) and A-3 (dotted line); (D) the peaks in (C) from A-4 (solid line) and from A-3 (dotted line) digested with diplococcal β-*N*-acetylhexosaminidase in the presence of γ-D-galactonolacton (1 mg/50 μL); (E) Smith degradation products of A-1; (F) Smith degradation product of A-2. Black triangles are the same as in Figure 2. Arrows indicate the elution position of authentic oligosaccharides: (a) GlcNAc$_2$Man$_2$GlcNAcXylNAc$_{OT}$; (b) GlcNAcMan$_2$GlcNAcXylNAc$_{OT}$; (c) Man$_2$GlcNAcXylNAc$_{OT}$; (d) ManGlcNAcXylNAc$_{OT}$; (e) GlcNAcXylNAc$_{OT}$.

11.0 glucose units was produced by the reaction (Figure 4C, solid line). This component was larger than the radioactive Smith degradation product of A-4N (Figure 4A) by 0.8 glucose unit. Therefore, one of the galactosyl residues, which was substituted by a sialic acid residue at the C-3 position and was resistant to periodate oxidation, should be linked to one of the *N*-acetylglucosamine residues of the GlcNAcβ1→4-(GlcNAcβ1→2)Manα1→3 group. Another NeuAcα2→3Gal group in A-4 should be linked to the GlcNAcβ1→2Manα1→6 side and lost as a fragment, Galβ1→4GlcNAcβ1→2-glycerol by Smith degradation. By diplococcal β-*N*-acetylhexosaminidase digestion, one *N*-acetylglucosamine residue was released from the solid line radioactive component in Figure 4C (Figure 4D, solid line). This result indicated that the GlcNAcβ1→2 residue of the GlcNAcβ1→4(GlcNAcβ1→-2)Manα1→3 group was not substituted by a β-galactosyl residue. Accordingly, the NeuAcα2→3Gal group should be exclusively located on the GlcNAcβ1→4Manα1→3 side. On the basis of a series of experimental results, the structure of A-4 was proposed as shown in Figure 5.

Structural Analysis of A-1, A-2, and A-3. A-1N, A-2N, and A-3N showed the same elution profiles on Bio-Gel P-4 as already described above, suggesting that these oligosaccharides have a common structure. Actually, sequential exoglycosidase digestion confirmed this presumption. Therefore, only the results of the structural analyses of A-3N will be described here.

Sequential digestion of A-3N with jack bean β-galactosidase and diplococcal or jack bean β-*N*-acethylhexosaminidases gave

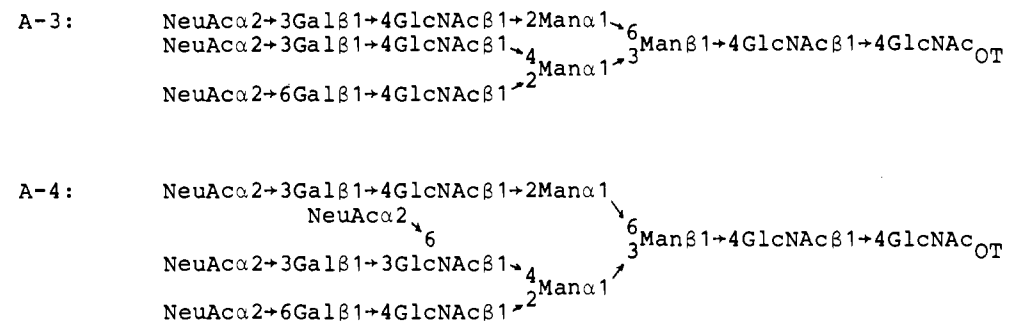


FIGURE 5: Proposed structures of fully sialylated oligosaccharides A-3 and A-4.

exactly the same results as those for A-4N. However, all three galactosyl residues were released from A-3N by diplococcal β-galactosidase digestion (not shown). On the basis of these results and the methylation data (Table I), a 2,4-branched triantennary structure containing only Galβ1→4GlcNAc outer chains was proposed for A-3N. The location of the 2,4 branch was analyzed by two cycles of Smith degradation of A-3N. Degradation products showed entirely the same elution profiles (not shown) as those of A-4N which were shown in Figure 4A,B. Therefore, it was concluded that the 2,4 branch is exclusively located on Manα1→3 side, supporting the data obtained by Nilsson et al. (1979).

As to the linkage and the location of *N*-acetylneuraminic acid residues, the following data were obtained. (1) Two residues of 2,4,6-tri-*O*-methyl derivatives and one residue of 2,3,4-tri-*O*-methyl derivatives of galactose were newly detected from A-3 by the methylation study (Table I), indicating the presence of NeuAcα2→3Gal and NeuAcα2→6Gal in a ratio of 2 to 1. (2) Smith degradation of radioactive A-3 gave a single radioactive component which is eluted slightly faster than the product from radioactive A-4 (Figure 4C, dotted line). An *N*-acetylglucosamine residue was released from the radioactive component by diplococcal β-*N*-acetylhexosaminidase treatment (Figure 4D, dotted line). These results indicated that a NeuAcα2→3Gal sequence is located on the GlcNAcβ1→4 side and a NeuAcα2→6Gal on the GlcNAcβ1→2 side of the 2,4 branch. As already reported (Yamashita et al., 1982), the effective size of the Galβ1→3GlcNAc group is slightly smaller than that of the Galβ1→4GlcNAc group in Bio-Gel P-4 column chromatography. On the basis of these results, the structure of A-3 was proposed as shown in Figure 5.

A-1 and A-2 are mono- and disialyl derivatives, respectively, of the undecasaccharide A-3N. In view of the evidence of selective sialylation reported by Van den Ijnden et al. (1980), the locations of sialic acid residues in these partially sialylated oligosaccharides were of interest. In order to determine the location of an *N*-acetylneuraminic acid residues, radioactive A-1 was subjected to the following analyses. Two nonreducing terminal galactosyl residues were removed from A-1 by jack bean β-galactosidase digestion, and the resultant degalactosylated A-1 was subjected to Smith degradation. As shown in Figure 4E, the product was eluted as two major peaks (I and II) and one minor peak (III). All three radioactive products should be derived from the 2,4-branched Manα1→3 arm. Since digestion of peak I with a mixture of diplococcal β-galactosidase and diplococcal β-*N*-acetylhexosaminidase released only one residue of galactose (data not shown), it should be a hexasaccharide, Galβ1→4GlcNAcβ1→4Manα1→3Manβ1→4GlcNAcβ1→4XylNAc$_{OT}$. This hexasaccharide should be obtained if the NeuAcα2→3Gal sequence is located on the GlcNAcβ1→4Manα1→3 arm. Since an *N*-acetylglucosaminyl residue was removed from peak II by diplococcal β-*N*-acetylhexosaminidase digestion (data not shown), it should be a pentasaccharide, GlcNAcβ1→2Manα1→3Manβ1→4GlcNAcβ1→4XylNAc$_{OT}$. Therefore, peak II must be produced from an oligosaccharide which has the NeuAcα2→6Gal sequence on a GlcNAcβ1→2Manα1→3 arm. Peak III was a tetrasaccharide, Manα1→3Manβ1→4GlcNAcβ1→4XylNAc$_{OT}$, originating from the oligosaccharide with an *N*-acetylneuraminic acid residue on its Manα1→6 arm. The percent molar ratio of peaks I, II, and III was 33, 60, and 7, respectively. These data indicated that sialylation occurs at all three sites, but preferentially on the Manα1→3 side.

The location of sialic acid residues in A-2 was analyzed similarly. As shown in Figure 4F, the Smith degradation product of degalactosylated A-2 was separated into three peaks, I′, II′, and III′, in a percent molar ratio of 48, 14, and 38, respectively. All the radioactive products should contain the Manα1→3 arm, since the Manα1→6 arm is released as unlabeled fragments. Peak I′ was eluted at the same position as the radioactive Smith degradation product of A-3 (Figure 4C, dotted line), the structure of which was elucidated as Galβ1→4GlcNAcβ1→4(GlcNAcβ1→2)Manα1→3Manβ1→4GlcNAcβ1→4XylNAc$_{OT}$. This result indicated that the product was produced from an oligosaccharide, in which the two outer chains of 2,4-branched Manα1→3 arm are fully sialylated and the outer chain on the Manα1→6 arm is degalactosylated. Actually, peak I′ was converted to GlcNAcβ1→4(GlcNAcβ1→2)Manα1→3Manβ1→4GlcNAcβ1→4XylNAc$_{OT}$ with release of a galactosyl residue by jack bean β-galactosidase digestion (data not shown). By the same series of analyses as described in the case of peaks I and II in Figure 4E, the structures of peaks II′ and III′ were elucidated as Galβ1→4GlcNAcβ1→4Manα1→3Manβ1→4GlcNAcβ1→4XylNAc$_{OT}$ and GlcNAcβ1→2Manα1→3Manβ1→4GlcNAcβ1→4XylNAc$_{OT}$, respectively (data not shown). Therefore, peaks II′ and III′ should be produced from the two radioactive oligosaccharides in which the Galβ1→4GlcNAcβ1→2Manα1→3 group and the Galβ1→4GlcNAcβ1→4Manα1→3 group were degalactosylated, respectively. A series of experimental results, so far described, indicated that the two *N*-acetylneuraminic acid residues of the oligosaccharides in A-2 were also distributed in all three possible combinations.

Structural Analyses of A-1′ and A-2′. A-1′ and A-2′ are minor components that accounted for 8% of total oligosaccharides in all. Structural analyses of A-1′N and A-2′N by sequential glycosidase digestion revealed that these sugar chains have biantennary structures (not shown). Almost equal amounts of 2,3,4-tri-*O*-methyl- and 2,4,6-tri-*O*-methylgalactitols were detected by the methylation analysis of A-2′ (not shown), indicating the presence of a NeuAcα2→6Gal and a NeuAcα2→3Gal in the outer chain moieties. By analogy with A-3, it is likely that NeuAcα2→6 is located on

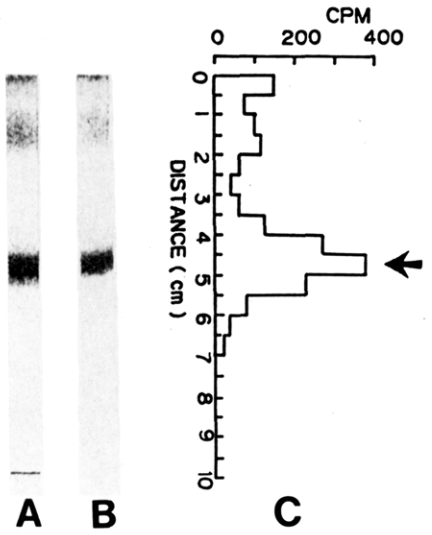


FIGURE 6: SDS–PAGE of purified fetuin: (A) gel stained with Coomassie brilliant blue; (B) gel stained with periodic acid–Schiff's reagent; (C) radioelectrophoretogram of sialic acid labeled fetuin preparation. An arrow indicates the position corresponding to the molecular weight (48 000) of fetuin.

Manα1→3 side, and NeuAcα2→3 on the Manα1→6 side, although we do not have any direct evidence. No further analysis was performed on these components because of the limited amount of the samples available.

Origin of A-4. As mentioned above, it became apparent that our purified and commercial preparations of fetuin contain heterogeneous sugar chains. Since the structure of A-4 is quite different from that of the others, we carefully examined whether the occurrence of this sugar chain is due to microheterogeneity or contamination with other glycoproteins. When the purified fetuin preparation was subjected to SDS–PAGE, some minor contaminating proteins, which were also stained by periodic acid–Schiff's reagent, were detected in addition to a major fetuin band (Figure 6). Therefore, we prepared sialic acid labeled fetuin by sodium periodate oxidation followed by NaB^3H_4 reduction, and the preparation was subjected to electrophoresis (for details, see Materials and Methods). The labeled fetuin extracted from the gel, which contained approximately 80% of the total radioactivity, was subjected to hydrazinolysis, and the pattern of sugar chains was examined. As shown in Figure 7, its pattern on paper electrophoresis at pH 5.4 was quite similar to that shown in Figure 1. This result indicated that all sugar chains including A-4 were derived from fetuin.

DISCUSSION

In this paper, we showed that fetuin contains a variety of asparagine-linked sugar chains. These sugar chains are different in the branching structure and the degree of sialylation. The majority of the sugar chains (43%) had a typical 2,4-branched triantennary structure as reported by Nilsson et al. (1979). Considerable amounts of mono- (6%) and disialyl (25%) oligosaccharides sharing a common structure in the neutral portion with the major trisialyl oligosaccharide were detected. In addition, we found the presence of oligosaccharides with biantennary structure (8%) and tetrasialyl triantennary structure (17%). Such heterogeneity was observed in the commercial fetuin preparation as well as in our purified preparation. On the basis of the evidence that fetuin has three N-glycosylation sites (Spiro, 1973), the amount of each of these minor oligosaccharides is calculated to be less than 1 mol per mole of protein. Therefore, we suspected that

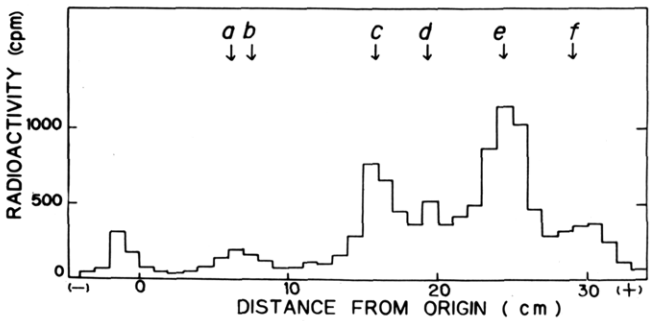


FIGURE 7: Paper electrophoretogram of radioactive oligosaccharides released from sialic acid labeled fetuin. The radioactive fetuin was extracted from the gel after SDS–PAGE of the sialic acid labeled fetuin and subjected to hydrazinolysis. The released oligosaccharides were analyzed by paper electrophoresis at pH 5.4 (80 V/cm, 2 h). Arrows a–f indicate the elution positions of A-1, A-1′, A-2, A-2′, A-3, and A-4 from fetuin, respectively.

the minor sugar chains might be derived from some minor glycoproteins contaminating the fetuin preparation. However, analysis of the sialic acid labeled fetuin, which was extracted from the gel after SDS–PAGE, indicated that all the oligosaccharides originated from the fetuin molecule. Thus, fetuin should be considered to be a mixture of molecules with different sets of three asparagine-linked sugar chains.

The disialyl tetrasaccharide sequence NeuAcα2→3Galβ1→3(NeuAcα2→6)GlcNAc included in the tetrasialyl oligosaccharides of fetuin has originally been found in the biantennary sugar chains of bovine prothrombin (Mizuochi et al., 1979), and then in those of bovine coagulation factor X (Mizuochi et al., 1980). Factor IX also contains that sequence in the triantennary sugar chains as well as in biantennary sugar chains. In the case of the triantennary sugar chains, the disialyl tetrasaccharide outer chain is located on the nonbranched GlcNAcβ1→2 side and the GlcNAcβ1→6 side of the 2,6 branch (Mizuochi et al., 1983). In contrast, the tetrasaccharide is located exclusively on the GlcNAcβ1→4 side of the 2,4 branch in the case of fetuin. Such a diverse location of the tetrasaccharide outer chain indicated that sialyltransferases involved in the formation of this unique structure have rather wide specificities for the branches. The fact that both NeuAcα2→3 and 6Galβ1→4GlcNAc outer chains are found in the sugar chains of fetuin and that only the NeuAcα2→6Galβ1→4GlcNAc outer chain is found in the blood coagulation factors is in good agreement with the enzymatic evidence reported by Van den Eijnden and Schiphorst (1981). They showed that fetal calf liver contains a Galβ1→4GlcNAcα2→6 sialyltransferase and a Galβ1→4GlcNAcα2→3 sialyltransferase, and the latter enzyme dramatically decreases during development of bovine liver.

Of interest is the selective location of *N*-acetylneuraminic acid residues in A-3, that the NeuAcα2→6Gal sequence was exclusively found on the GlcNAcβ1→2Manα1→3 side and that the NeuAcα2→3Gal sequences were on the GlcNAcβ1→2Manα1→6 side and the GlcNAcβ1→4Manα1→3 side. This selectivity might result from the difference in relative activities and branch specificities of the sialyltransferases. It is noteworthy that the α2→6 sialyltransferase preferentially transfers *N*-acetylneuraminic acid to the Galβ1→4GlcNAcβ1→2Manα1→3 sequence (Van den Eijnden et al., 1980).

During the preparation of this paper, Dr. J. F. G. Vliegenthart's group presented data that the Galβ1→3GlcNAc sequence occurred in a part of the triantennnary sugar chains of fetuin on the basis of ^{1}H NMR analysis (van Halbeek et al., 1985). The data of Vliegenthart's group also indicated

the presence of two isomeric forms of sialylation (the NeuAcα2→3Gal and the NeuAcα2→6Gal) on the Manα1→6 arm. We cannot rule out the presence of such isomers, since the Manα1→6 arm was lost during Smith degradation and the released fragments could not be directly analyzed. However, the methylation data suggested that the Manα1→6 arm is predominantly sialylated by an α2→3 linkage. Therefore, the NeuAcα2→6Gal, even if it is present, may occur as a minor component. By study on the interaction of hepatic Gal/GalNAc lectin with fetuin glycopeptides, Dr. Y. C. Lee's group also found the heterogeneity of fetuin glycopeptides and reached the same conclusion from ^{1}H NMR analysis (Townsend et al., 1986).

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REFERENCES

Baenziger, J. U., & Fiete, D. (1979) *J. Biol. Chem. 254*, 789–795.

Endo, Y., Yamashita, K., Tachibana, Y., Tojo, S., & Kobata, A. (1979) *J. Biochem. (Tokyo) 85*, 669–679.

Fairbanks, G., Steck, T. L., & Wallach, D. F. H. (1971) *Biochemistry 10*, 2606–2617.

Kobata, A., & Takasaki, S. (1978) *Methods Enzymol. 50*, 560–567.

Krusius, T., & Finne, J. (1981) *Carbohydr. Res. 90*, 203–214.

Laemmli, U. K. (1970) *Nature (London) 227*, 680–685.

Lenten, L. V., & Ashwell, G. (1972) *Methods Enzymol. 28*, 209–211.

Li, Y.-T., & Li, S.-C. (1972) *Methods Enzymol. 28*, 702–713.

Mizoguchi, A., Takasaki, S., Maeda, S., & Kobata, A. (1984a) *J. Biol. Chem. 259*, 11943–11948.

Mizoguchi, A., Takasaki, S., Maeda, S., & Kobata, A. (1984b) *J. Biol. Chem. 259*, 11949–11957.

Mizuochi, T., Yamashita, K., Fujikawa, K., Kisiel, W., & Kobata, A. (1979) *J. Biol. Chem. 254*, 6419–6425.

Mizuochi, T., Yamashita, K., Fujikawa, K., Titani, K., & Kobata, A. (1980) *J. Biol. Chem. 255*, 3526–3531.

Mizuochi, T., Taniguchi, T., Fujikawa, K., Titani, K., & Kobata, A. (1983) *J. Biol. Chem. 258*, 6020–6024.

Nilsson, B., Norden, N. E., & Svensson, S. (1979) *J. Biol. Chem. 254*, 4545–4553.

Paulson, J. C., Prieels, J.-P., Galsgow, L. R., & Hill, R. L. (1978) *J. Biol. Chem. 253*, 5617–5624.

Spik, G., Bayand, B., Fournet, B., Strecker, G., Bouquelet, S., & Montreuil, J. (1975) *FEBS Lett. 50*, 296–299.

Spiro, R. G. (1960) *J. Biol. Chem. 235*, 2860–2869.

Spiro, R. G. (1962) *J. Biol. Chem. 237*, 382–388.

Spiro, R. G., & Bhoyroo, V. D. (1974) *J. Biol. Chem. 249*, 5704–5717.

Takasaki, S., Mizuochi, T., & Kobata, A. (1982) *Methods Enzymol. 83*, 263–268.

Takasaki, S., Murray, G. J., Furbish, F. S., Brady, R. O., Barranger, J. A., & Kobata, A. (1984) *J. Biol. Chem. 259*, 10112–10117.

Townsend, R. R., Hardy, M. R., Wong, T. C., & Lee, Y. C. (1986) *Biochemistry* (following paper in this issue).

Uchida, Y., Tsukada, Y., & Sugimor, T. (1974) *Biochim. Biophys. Acta 350*, 425–431.

Van den Eijnden, D. H., & Schiphorst, W. E. C. M. (1981) *J. Biol. Chem. 256*, 3159–3162.

Van den Eijnden, D. H., Joziasse, D. H., Dorland, L., Van Halbeek, H., Vliegenthart, J. F. G., & Schmid, K. (1980) *Biochem. Biophys. Res. Commun. 92*, 839–945.

van Halbeek, H., Rijkse, I., van Beek, W. P., Kamerling, J. P., & Vliegenthart, J. F. G. (1985) in *Glycoconjugates: Proceedings of the VIIIth International Symposium* (Davidson, E. A., Williams, J. C., & Di Ferrante, N. M., Eds.) pp 120–121, Praeger, New York.

Yamashita, K., Liang, C.-J., Funakoshi, S., & Kobata, A. (1981a) *J. Biol. Chem. 256*, 1283–1289.

Yamashita, K., Ohkura, T., Yoshima, H., & Kobata, A. (1981b) *Biochem. Biophys. Res. Commun. 100*, 226–232.

Yamashita, K., Mizuochi, T., & Kobata, A. (1982) *Methods Enzymol. 83*, 105–126.

Yoshima, H., Takasaki, S., & Kobata, A. (1980) *J. Biol. Chem. 255*, 10793–10804.