tyrosine at residue 16. Consequently, the most likely explanation for this observation is the coisolation of the β chains of HLA-DR antigens with that of a structurally very similar human Ia antigen that is phenotypically distinct from HLA-DR.

The two regions of high polymorphism in the β chains, residues 7–13 and 26–31, are of adequate length to represent distinct antigenic sites since it has been reported that a hexapeptide is approximately the size of an antigenic determinant (Atassi, 1975). We are currently synthesizing these two regions of the molecule and are attempting to produce both polyclonal and monoclonal antibodies reactive with them to determine whether these two regions of amino acid sequence variability are important for the recognition of allospecific sites. The seemingly high degree of structural polymorphism that exists among the β chains of HLA-DR1 and HLA-DR2 antigens is somewhat surprising in light of a report by Kratzin et al. (1981), who showed that an antigen preparation isolated from a homozygous B lymphoid cell line (HLA-DR2,2) is actually comprised of a pool of seven β chains which are highly variable in only one five amino acid region (residues 65–69) of each molecule. If, as suggested by Kratzin et al. (1981), these β chains are indeed the product of a cluster of different genes, then the structural difference between two HLA-DR allospecificities (HLA-DR1 and HLA-DR2) are more numerous than those detected between HLA-DR and other human Ia gene products.

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Carbohydrates of Influenza Virus Hemagglutinin: Structures of the Whole Neutral Sugar Chains†

Akira Matsumoto, Hideo Yoshima, and Akira Kobata*

ABSTRACT: The carbohydrates of BHA, a solubilized hemagglutinin of influenza virus by bromelain digestion, were quantitatively released as oligosaccharides by hydrazinolysis. The oligosaccharide mixture was separated into a neutral and two acidic fractions by paper electrophoresis. Both acidic fractions were resistant to sialidase digestion but were slowly converted to the neutral fraction by incubation with sulfatases. The neutral fraction which comprised about 80% in molar ratio of total oligosaccharides was separated into 13 oligosaccharides by paper chromatography and by Con A–Sepharose column chromatography. Structural studies of these oligosaccharides by sequential exoglycosidase digestion and by methylation analysis revealed that BHA contains a series of high mannose type and bi-, tri-, and tetraantennary complex type sugar chains. Occurrence of Galβ1→3GlcNAc outer chain in two and bisectional *N*-acetylglucosamine in one of the biantennary sugar chains is an interesting characteristic of the sugar chains of BHA.

Among the proteins coded by influenza virus, the hemagglutinin and the neuraminidase are integral membrane glycoproteins which construct spikes on the viral envelope. When hemagglutinin (HA) binds specifically to the sialated components of host cell plasma membranes, HA adsorbs virus particles to the cell surface and induces penetration of viral RNA into the cell by fusing the viral envelope with the cellular membrane. The hemagglutinin is also the major surface antigen of the virus, and variations in its antigenic structure accompany the recurrences of influenza epidemics in man.

The HA of A2/Hong Kong/1968 virus is a trimer of molecular weight of about 220000 (Wiley & Skehel, 1977), and each subunit of the glycoprotein is composed of two di-

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sulfide-linked polypeptide chains called HA1 and HA2. The HA can be removed from virus particles by bromelain digestion, and the soluble glycoprotein (BHA) thus obtained lacks the membrane anchoring C-terminal peptide of the subunits (Skehel & Waterfield, 1975) and has been crystallized. The three-dimensional structure of BHA isolated from X-31 (H_3N_2) virus has recently been elucidated by X-ray crystallography (Wilson et al., 1981), and this paper will describe structural studies of the whole carbohydrate moieties of BHA obtained from the X-31 virus.

Experimental Procedures

Preparation of Bromelain-Released Hemagglutinin (BHA). BHA was obtained by bromelain digestion of X-31 strain of A2/Hong Kong/1968 virus and purified according to the method of Brand & Skehel (1972). As reported previously (Skehel & Waterfield, 1975), the BHA contains all carbohydrate moiety of intact HA and consists of two types of glycopeptide of apparent molecular weights 58 000, HA1, and 21 000, HA2, which can be separated by sodium dodecyl sulfate ($NaDodSO_4$)–polyacrylamide gel electrophoresis in the presence of β-mercaptoethanol.

Oligosaccharides. Manα1→6(Manα1→3)Manα1→6-(Manα1→3)Manβ1→4GlcNAcβ1→4GlcNAc$_{OT}$ (Man$_5$·GlcNAc·GlcNAc$_{OT}$),[1] (Manα1→2)·Man$_5$·GlcNAc·GlcNAc$_{OT}$ (Man$_6$·GlcNAc·GlcNAc$_{OT}$), and (Manα1→2)$_2$·Man$_5$·GlcNAc·GlcNAc$_{OT}$ (Man$_7$·GlcNAc·GlcNAc$_{OT}$) were obtained by hydrazinolysis of bovine pancreatic ribonuclease B (Liang et al., 1980). Manβ1→4GlcNAcβ1→4GlcNAc$_{OT}$ (Man·GlcNAc·GlcNAc$_{OT}$) and GlcNAcβ1→4GlcNAc$_{OT}$ were obtained by sequential digestion of Man$_5$·GlcNAc·GlcNAc$_{OT}$ with jack bean α-mannosidase and β-mannosidase, respectively. *N*-Acetyl[^{3}H]glucosaminitol was obtained by NaB^3H_4 reduction of *N*-acetylglucosamine (Takasaki & Kobata, 1978). Galβ1→4GlcNAcβ1→2Manα1→6(Galβ1→4GlcNAcβ1→-2Manα1→3)Manβ1→4GlcNAcβ1→4(Fucα1→6)GlcNAc$_{OT}$ (Gal$_2$·GlcNAc$_2$·Man$_3$·GlcNAc·Fuc·GlcNAc$_{OT}$) was obtained from subcomponent C1q of the first component of human complement (Mizuochi et al., 1978). GlcNAcβ1→-2Manα1→6(GlcNAcβ1→2Manα1→3)Manβ1→-4GlcNAcβ1→4(Fucα1→6)GlcNAc$_{OT}$ (GlcNAc$_2$·Man$_3$·GlcNAc·Fuc·GlcNAc$_{OT}$), Manα1→6(Manα1→3)Manβ1→-4GlcNAcβ1→4(Fucα1→6)GlcNAc$_{OT}$ (Man$_3$·GlcNAc·Fuc·GlcNAc$_{OT}$), Manβ1→4GlcNAcβ1→4(Fucα1→6)GlcNAc$_{OT}$ (Man·GlcNAc·Fuc·GlcNAc$_{OT}$), and GlcNAcβ1→4(Fucα1→-6)GlcNAc$_{OT}$ (GlcNAc·Fuc·GlcNAc$_{OT}$) were obtained by sequential digestion of Gal$_2$·GlcNAc$_2$·Man$_3$·GlcNAc·Fuc·GlcNAc$_{OT}$ with jack bean β-galactosidase, jack bean β-*N*-acetylhexosaminidase, jack bean α-mannosidase, and β-mannosidase, respectively. Fucα1→6GlcNAc$_{OT}$ was isolated from the urine of fucosidosis patients (Nishigaki et al., 1978). Manα1→6(Galβ1→4GlcNAcβ1→2Manα1→3)Manβ1→-4GlcNAcβ1→4(Fucα1→6)GlcNAc$_{OT}$ (Gal·GlcNAc·Man$_3$·GlcNAc·Fuc·GlcNAc$_{OT}$), Galβ1→4GlcNAcβ1→4(Galβ1→-4GlcNAcβ1→2)Manα1→6(Galβ1→4GlcNAcβ1→-2Manα1→3)Manβ1→4GlcNAcβ1→4(Fucα1→6)GlcNAc$_{OT}$ (Gal$_3$·GlcNAc$_3$·Man$_3$·GlcNAc·Fuc·GlcNAc$_{OT}$), and Galβ1→-4GlcNAcβ1→4(Galβ1→4GlcNAcβ1→2)Manα1→6-[Galβ1→4GlcNAcβ1→6(Galβ1→4GlcNAcβ1→2)Manα1→-3]Manβ1→4GlcNAcβ1→4(Fucα1→6)GlcNAc$_{OT}$ (Gal$_4$·GlcNAc$_4$·Man$_3$·GlcNAc·Fuc·GlcNAc$_{OT}$) were obtained from calf thymocyte plasma membrane (Yoshima et al., 1980b). GlcNAcβ1→4(GlcNAcβ1→2)Manα1→6(GlcNAcβ1→-2Manα1→3)Manβ1→4GlcNAcβ1→4(Fucα1→6)GlcNAc$_{OT}$ (GlcNAc$_3$·Man$_3$·GlcNAc·Fuc·GlcNAc$_{OT}$) and GlcNAcβ1→-4(GlcNAcβ1→2)Manα1→6[GlcNAcβ1→6(GlcNAcβ1→-2)Manα1→3]Manβ1→4GlcNAcβ1→4(Fucα1→6)GlcNAc$_{OT}$ (GlcNAc$_4$·Man$_3$·GlcNAc$_{OT}$) were obtained by jack bean β-galactosidase digestion of Gal$_3$·GlcNAc$_3$·Man$_3$·GlcNAc·Fuc·GlcNAc$_{OT}$ and Gal$_4$·GlcNAc$_4$·Man$_3$·GlcNAc·Fuc·GlcNAc$_{OT}$, respectively. Galβ1→4GlcNAcβ1→2Manα1→-6(Galβ1→4GlcNAcβ1→2Manα1→3)(GlcNAcβ1→4)-Manβ1→4GlcNAcβ1→4(Fucα1→6)GlcNAc$_{OT}$ (Gal$_2$·GlcNAc$_2$·GlcNAc·Man$_3$·GlcNAc·Fuc·GlcNAc$_{OT}$) and GlcNAcβ1→2Manα1→6(GlcNAcβ1→2Manα1→3)-(GlcNAcβ1→4)Manβ1→4GlcNAcβ1→4(Fucα1→6)-GlcNAc$_{OT}$ (GlcNAc$_2$·GlcNAc·Man$_3$·GlcNAc·Fuc·GlcNAc$_{OT}$) were obtained from glycophorin A (Yoshima et al., 1980a). Galβ1→4(Fucα1→3)GlcNAcβ1→2Manα1→6[Galβ1→4-(Fucα1→3)GlcNAcβ1→2Manα1→3]Manβ1→4GlcNAc-β1→4(Fucα1→6)GlcNAc$_{OT}$ (Gal$_2$·Fuc$_2$·GlcNAc$_2$·Man$_3$·GlcNAc·Fuc·GlcNAc$_{OT}$) and a mixture of Galβ1→4-(Fucα1→3)GlcNAcβ1→2Manα1→(6- or -3)[Galβ1→-4GlcNAcβ1→2Manα1→(3- or -6)]Manβ1→4GlcNAcβ1→-4(Fucα1→6)GlcNAc$_{OT}$ (Gal$_2$·Fuc·GlcNAc$_2$·Man$_3$·GlcNAc·Fuc·GlcNAc$_{OT}$) were obtained from human parotid α-amylase (Yamashita et al., 1980b).

Chemicals and Enzymes. NaB^3H_4 (246 mCi/mmol) was purchased from New England Nuclear, Boston, MA. NaB^2H_4 (98%) was purchased from Merck Co., Inc., Darmstadt, F. R.G. β-Galactosidase, β-*N*-acetylhexosaminidase, and α-mannosidase were purified from jack bean meal by the method of Li & Li (1972). β-Galactosidase and β-*N*-acetylhexosaminidase were also purified from the culture medium of *Diplococcus pneumoniae* by the method of Glasgow et al. (1977).

Diplococcal β-galactosidase hydrolyzes Galβ1→4GlcNAc linkage but not Galβ1→3GlcNAc and Galβ1→6GlcNAc linkages (Paulson et al., 1978). Diplococcal β-*N*-acetylhexosaminidase also has a useful substrate specificity for the structural study of asparagine-linked sugar chains (Yamashita et al., 1981): The enzyme hydrolyzes the GlcNAcβ1→2Man linkage but not GlcNAcβ1→4Man and GlcNAcβ1→6Man linkages at low substrate concentration. The GlcNAcβ1→-2Man linkage in the GlcNAcβ1→4(GlcNAcβ1→2)Man group is cleaved by the enzyme, but the linkage in GlcNAcβ1→6(GlcNAcβ1→2)Man group is not cleaved. α-Mannosidase of *Aspergillus saitoi* was purified by the method of Ichishima et al. (1981). This enzyme hydrolyzes the Manα1→2Man linkage but not Manα1→3Man and Manα1→6Man linkages (Yamashita et al., 1980a) and is a useful reagent for identifying the high mannose type oligosaccharides (Mizuochi et al., 1981). Snail β-mannosidase (Sugawara et al., 1972) and *Charonia lampas* α-fucosidase (Nishigaki et al., 1974) were kindly supplied by Seikagaku Kogyo Co., Tokyo. α-Fucosidase I was purified from almond emulsin as reported previously (Yoshima et al., 1979). Another α-fucosidase was purified from *Bacillus fulminans* according to the method of Kochibe (1973). These fucosidases have different substrate specificities: the *Charonia lampas* enzyme hydrolyzes all α-fucosyl linkages so far reported, while the *Bacillus* enzyme cleaves the Fucα1→2Gal linkage only and the almond enzyme cleaves specifically the α-fucosyl linkages in Galβ1→4(Fucα1→3)GlcNAc—and Galβ1→3-(Fucα1→4)GlcNAc—groups. Sulfatases from limpets (type V) and abalone (type VII) were purchased from Sigma Chemical Co., St. Louis, MO.

[1] Subscript OT is used in this paper to indicate NaB^3H_4-reduced sugars. All sugars mentioned in this paper were of D configuration, except for fucose which had an L configuration.

Analytical Methods. High-voltage paper electrophoresis was performed with either pyridine/acetate buffer, pH 5.4 (pyridine/acetic acid/water, 3:1:387), at a potential of 89 V/cm for 1.5 h, or 0.06 M borate buffer, pH 9.5, at 40 V/cm for 5.5 h. Descending paper chromatography was performed with ethyl acetate/pyridine/acetic acid/water (5:5:1:3). Radioactivity was determined on an Aloka liquid scintillation spectrometer, Model LSC-700. Radiochromatoscanning was performed with a Packard radiochromatogram scanner, Model 7201. Bio-Gel P-4 (smaller than 400 mesh) column chromatography was performed as reported in the previous paper (Yamashita et al., 1982) by using a 2-m column. Methylation analysis of oligosaccharides was performed as described in the previous paper (Endo et al., 1979). Concanavalin A (Con A)–Sepharose column chromatography was performed as follows. A column (0.5 × 5 cm) of Con A–Sepharose was equilibrated with 0.01 M tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl) buffer, pH 7.5, containing 0.1 M NaCl. Radioactive oligosaccharides in less than 0.1 mL of the buffer were applied to the column, and the column was washed with 10 mL of the buffer. The column was then washed with 10 mL of the buffer containing 0.1 M methyl α-mannopyranoside. Fractions of 1 mL were collected, and the radioactivity in each tube was determined on an aliquot of sample by the liquid scintillation method. As reported by Ogata et al. (1975), complex-type sugar chains in which the two α-mannosyl residues of the trimannosyl core are either free or substituted only at the C-2 position by outer chains bind to the column and are eluted by 0.1 M methyl α-mannopyranoside solution. Periodate oxidation of radioactive oligosaccharides was performed as reported previously (Yoshima et al., 1981).

Enzymatic Digestion of Oligosaccharides. Radioactive oligosaccharides [(1–2) × 10^4 cpm] were incubated with one of the following mixtures at 37 °C for 18 h: (1) *jack bean β-galactosidase digestion*, enzyme (0.7 unit) in 0.1 M citrate/phosphate buffer, pH 3.5 (50 μL); (2) *jack bean β-N-acetylhexosaminidase digestion*, enzyme (0.7 unit) in 0.1 M citrate/phosphate buffer, pH 5.0 (50 μL); (3) *diplococcal β-galactosidase digestion*, enzyme (20 milliunits) in 0.1 M citrate/phosphate buffer, pH 6.0 (50 μL); (4) *diplococcal β-N-acetylhexosaminidase digestion*, enzyme (4 milliunits) in 0.1 M citrate/phosphate buffer, pH 6.0 (50 μL); (5) *jack bean α-mannosidase digestion*, enzyme (0.5 unit) in 0.1 M acetate buffer, pH 4.5 (50 μL); (6) *Aspergillus α-mannosidase digestion*, enzyme (0.15 μg) in 0.1 M acetate buffer, pH 5.0 (30 μL); (7) *β-mannosidase digestion*, enzyme (20 milliunits) in 0.5 M sodium citrate buffer, pH 4.5 (30 μL); (8) *Charonia lampas α-fucosidase digestion*, enzyme (0.2 unit) in 0.1 M acetate buffer, pH 4.0, containing 0.1 M NaCl (60 μL); (9) *almond α-fucosidase I digestion*, enzyme (40 microunits) in 0.1 M citrate/phosphate buffer, pH 5.0 (50 μL); (10) *Bacillus α-fucosidase digestion*, enzyme (17.5 μg) in 0.05 M sodium phosphate buffer, pH 6.6 (30 μL); (11) *diplococcal β-galactosidase and diplococcal β-N-acetylhexosaminidase digestion*, β-galactosidase (20 milliunits) and β-*N*-acetylhexosaminidase (4 milliunits) in 0.1 M citrate/phosphate buffer, pH 6.0 (50 μL). Fifty microliters of toluene was added to all reaction mixtures to inhibit bacterial growth. Reactions were terminated by heating the reaction mixture in a boiling water bath for 2 min, and the products were analyzed by Bio-Gel P-4 column chromatography.

Release of the Carbohydrate Moieties of BHA. BHA (37 mg) was suspended in 0.5 mL of anhydrous hydrazine and subjected to 10-h hydrazinolysis as reported in the previous paper (Takasaki et al., 1982). The oligosaccharide fraction thus obtained was dissolved in 0.5 mL of 0.05 N NaOH. To one-fifth of the solution was added 10 nmol of lactose as an internal standard, and the sugars in the solution were reduced with NaB^3H_4 (1.5 mCi) as reported previously (Takasaki & Kobata, 1974). [^{3}H]Lactitol and tritium-labeled oligosaccharide fractions were separated by paper chromatography by using solvent I. The yield of total radioactive oligosaccharides was 2.2 × 10^7 cpm. On the basis of the specific activity of the NaB^3H_4 calculated from the radioactivity incorporated into lactitol and the molecular weight (79 000) of a BHA subunit, the sugar chains released from 1 mol of BHA was calculated as 6.5 mol. The remaining sample was reduced with 5 mg of NaB^2H_4 to obtain deuterium-labeled oligosaccharide mixture. For facilitation of detection of oligosaccharides in further purification procedures, half of the tritium-labeled oligosaccharide mixture was added to the deuterium-labeled sample.

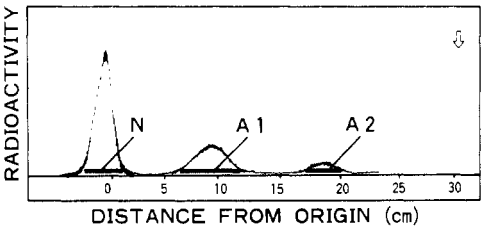


FIGURE 1: Paper electrophoretogram of oligosaccharides liberated from X-31 BHA glycoprotein by hydrazinolysis. After reduction with NaB^3H_4, the oligosaccharide mixture was subjected to paper electrophoresis at pH 5.4. Arrow indicates the position to which bromophenol blue migrated.

Results

Fractionation of Oligosaccharides by Paper Electrophoresis. The radioactive oligosaccharide fraction obtained from X-31 BHA glycoprotein by hydrazinolysis was spotted on Whatman 3MM paper and subjected to paper electrophoresis at pH 5.4. As shown in Figure 1, it was separated into a neutral (N) and two acidic (A1 and A2) fractions. These three fractions were recovered from paper by elution with water. The percent molar ratio of N, A1, and A2 calculated on the basis of their radioactivities was 79:16:5.

Aliquots (1.5 × 10^4 cpm) of N, A1, and A2 were hydrolyzed in 0.4 mL of 4 N HCl at 100 °C for 2 h, and the reaction mixture was freed from HCl by repeated evaporation with H_2O. The hydrolysates were N-acetylated and analyzed by paper electrophoresis using borate buffer as reported previously (Takasaki & Kobata, 1978). *N*-Acetylglucosaminitol was the only radioactive component detected from the three fractions. Therefore, *N*-acetylglucosamine is located at the reducing termini of all oligosaccharides in the three fractions as expected from the hydrazinolysis reaction of asparagine-linked sugar chains (Takasaki et al., 1982).

Both A1 and A2 were completely resistant to sialidase digestion. When A1 and A2 were incubated with sulfatases, they were slowly converted to neutral components. These results indicated that the acidic nature of A1 and A2 cannot be ascribed to sialic acid like most other asparagine-linked sugar chains but to sulfate residues as suggested by Printer & Compans (1975).

Fractionation of Neutral Oligosaccharide by Paper Chromatography. When the N fraction was subjected to paper chromatography for 7 days, a chromatogram shown in Figure 2A was obtained. Fractions N3 and N4 as indicated by white bars in Figure 2A were recovered from paper by elution with H_2O and subjected to the same paper chromatography for 28

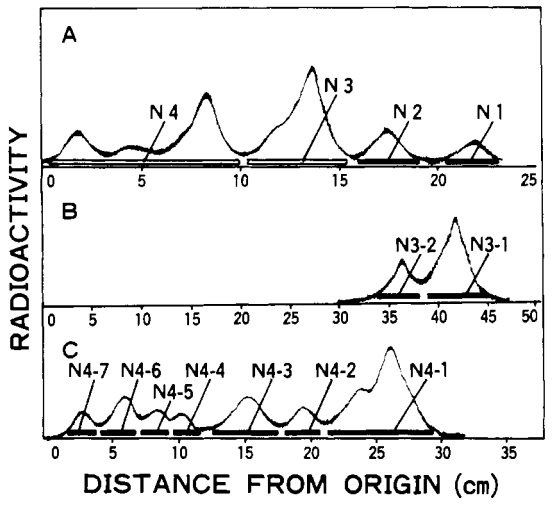


FIGURE 2: Fractionation of neutral oligosaccharides by paper chromatography. (A) N fraction obtained by paper electrophoresis was subjected to descending paper chromatography by using solvent I for 7 days; (B) fraction N3 in (A) was subjected to rechromatography with the same solvent for 28 days; (C) fraction N4 in (A) was subjected to rechromatography with the same solvent for 28 days.

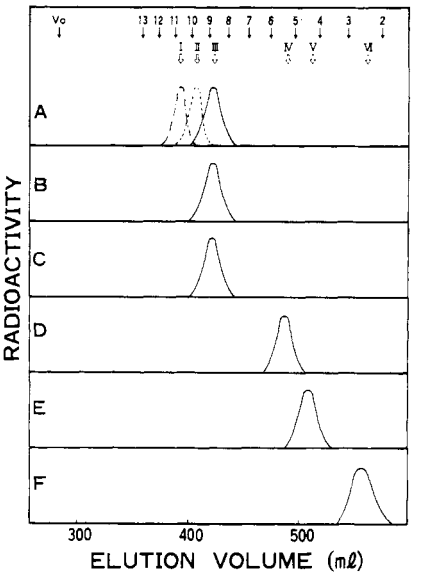


FIGURE 3: Sequential exoglycosidase digestion of oligosaccharide N1, N2, N3-1, and N3-2. Black arrows indicate the eluting positions of glucose oligomers (*numbers* indicate the glucose units) and void volume (Vo). White arrows indicate the positions where authentic oligosaccharides eluted: I, Man_7·GlcNAc·$GlcNAc_{OT}$; II, Man_6·GlcNAc·$GlcNAc_{OT}$; III, Man_5·GlcNAc·$GlcNAc_{OT}$; IV, Man·GlcNAc·$GlcNAc_{OT}$; V, $GlcNAc\beta1\rightarrow4GlcNAc_{OT}$; VI, *N*-acetyl-[^{3}H]glucosaminitol. (A) Oligosaccharides N1 (—), N2 (---), and N3-1 or N3-2 (···); (B) oligosaccharide N3-1 incubated with jack bean β-*N*-acetylhexosaminidase; (C) oligosaccharides N2 and N3-2 incubated with *Aspergillus* α-mannosidase; (D) the radioactive peak in (B) or (C) incubated with jack bean α-mannosidase; (E) the radioactive peak in (D) incubated with β-mannosidase; (F) the radioactive peak in (E) incubated with jack bean β-*N*-acetylhexosaminidase.

days. As shown in Figure 2B,C, N3 was separated into two (N3-1 and N3-2) and N4 into at least seven (N4-1–N4-7) fractions. The 11 fractions in total as indicated by black bars in Figure 2A–C were recovered from papers. The percent molar ratio of N1, N2, N3-1, N3-2, N4-1, N4-2, N4-3, N4-4, N4-5, N4-6, and N4-7 calculated on the basis of their radioactivities was 9.8:13.9:34.5:15.2:13.0:2.2:4.2:1.5:2.1:2.3:1.7.

Structures of N1, N2, N3-1, and N3-2 Fractions. When N1, N2, N3-1, and N3-2 were analyzed by Bio-Gel P-4 column, they were eluted as single components. The mobilities of N1, N2, and N3-2 were the same as authentic Man_5·GlcNAc·$GlcNAc_{OT}$, Man_6·GlcNAc·$GlcNAc_{OT}$, and Man_7·GlcNAc·$GlcNAc_{OT}$, respectively (Figure 3A). When N2 and N3-2 were incubated with *Aspergillus* α-mannosidase, they were converted to a radioactive component with the same mobility as authentic Man_5·GlcNAc·$GlcNAc_{OT}$ releasing one and two mannose residues, respectively (Figure 3C). The mobility of N3-1 in the Bio-Gel P-4 column was the same as that of authentic Man_7·GlcNAc·$GlcNAc_{OT}$. However, this oligosaccharide was totally resistant to *Aspergillus* α-mannosidase digestion (data not shown). When incubated with jack bean β-*N*-acetylhexosaminidase, it was converted to a radioactive component with the same mobility as authentic Man_5·GlcNAc·$GlcNAc_{OT}$ releasing an *N*-acetylglucosamine residue (Figure 3B). That the structures of the radioactive components in Figure 3C are Man_5·GlcNAc·$GlcNAc_{OT}$ was confirmed by sequential exoglycosidase digestion: they were converted to Man·GlcNAc·$GlcNAc_{OT}$ and $GlcNAc\beta1\rightarrow4GlcNAc_{OT}$ by sequential digestion with jack bean α-mannosidase (Figure 3D) and β-mannosidase (Figure 3E), respectively, and finally converted to *N*-acetyl[^{3}H]glucosaminitol by jack bean β-*N*-acetylhexosaminidase digestion (Figure 3F). These results indicated that N1, N2, and N3-2 are a series of high mannose type sugar chains, and N3-1 is one of the processing intermediates as shown in Figure 8. Because of the limited amount of sample available, methylation analysis of these oligosaccharides was not performed.

Structures of N4-1, N4-2, and N4-3 Fractions. When subjected to Con A–Sepharose column chromatography, 68% of N4-1 fraction bound to the column and eluted with methyl α-mannopyranoside solution, while N4-2 and N4-3 fractions did not bind to the column. The fraction of N4-1 bound to the column will be called in this paper as N4-1b and that unbound as N4-1u.

N4-1b, N4-2, and N4-3 gave single peaks upon Bio-Gel P-4 column chromatography (Figure 4A). When incubated with jack bean β-galactosidase, 1 and 2 mol of galactose were released from N4-2 and N4-1b, respectively, while N4-3 remained unchanged (Figure 4B). The radioactive products in Figure 4B from N4-2 and N4-1b released 1 and 2 mol of *N*-acetylglucosamine residue by jack bean β-*N*-acetylhexosaminidase digestion (Figure 4C), while N4-3 was still resistant to the enzyme digestion (data not shown). The mobility of the radioactive product from N4-1b at this stage was the same as that of authentic Man_3·GlcNAc·Fuc·$GlcNAc_{OT}$. That the component actually has the structure $Man\alpha1\rightarrow6(Man\alpha1\rightarrow3)Man\beta1\rightarrow4GlcNAc\beta1\rightarrow4(Fuc\alpha1\rightarrow6)GlcNAc_{OT}$ was confirmed by sequential exoglycosidase digestion documented in Figure 4D–G and methylation analysis in Table I. Therefore, the structure of N4-1b should be $Gal\beta1\rightarrow GlcNAc\beta1\rightarrow Man\alpha1\rightarrow6(Gal\beta1\rightarrow GlcNAc\beta1\rightarrow Man\alpha1\rightarrow3)Man\beta1\rightarrow4GlcNAc\beta1\rightarrow4(Fuc\alpha1\rightarrow6)GlcNAc_{OT}$. That the mobility of N4-1b in Bio-Gel P-4 column chromatography was the same as that of authentic Gal_2·$GlcNAc_2$·Man_3·GlcNAc·Fuc·$GlcNAc_{OT}$ (Figure 4A) also supports this assumption.

The mobilities of N4-2 and N4-3 in Bio-Gel P-4 column were the same as those of authentic Gal_2·Fuc·$GlcNAc_2$·Man_3·GlcNAc·Fuc·$GlcNAc_{OT}$ and Gal_2·Fuc_2·$GlcNAc_2$·Man_3·GlcNAc·Fuc·$GlcNAc_{OT}$, respectively. For confirmation of the presence of α-fucosyl residues in the outer chain moieties of these oligosaccharides, radioactive N4-2 and N4-3 were incubated with almond α-fucosidase I. As shown in Figure 4H, N4-2 released one and N4-3 released two fucose residues by this treatment. The mobilities of radioactive products from

Table 1: Methylation Analysis of N4-1b, N4-1u, N4-2, N4-3, and the Core Portion of N4-1b

partially methylated sugars	molar ratio[b]				
	N4-1b	N4-1u	N4-2	N4-3	N4-1b[a] (-2Gal and -2GlcNAc)
fucitol					
2,3,4-tri-*O*-methyl (1,5-di-*O*-acetyl)	1.3	1.4	2.3	3.3	1.0
galactitol					
2,3,4,6-tetra-*O*-methyl (1,5-di-*O*-acetyl)	2.4	2.3	2.3	2.2	0
mannitol					
2,3,4,6-tetra-*O*-methyl (1,5-di-*O*-acetyl)	0	0	0	0	2.3
3,4,6-tri-*O*-methyl (1,2,5-tri-*O*-acetyl)	2.3	2.2	2.4	2.1	0
2,4-di-*O*-methyl (1,3,5,6-tetra-*O*-acetyl)	*1.0*	0	*1.0*	*1.0*	*1.0*
2-mono-*O*-methyl (1,3,4,5,6-penta-*O*-acetyl)	0	*1.0*	0	0	0
2-(*N*-methylacetamido)-2-deoxyglucitol					
3,4,6-tri-*O*-methyl (1,5-di-*O*-acetyl)	0	0.6	0	0	0
3,6-di-*O*-methyl (1,4,5-tri-*O*-acetyl)	2.3	3.3	1.3	1.4	1.3
4,6-di-*O*-methyl (1,3,5-tri-*O*-acetyl)	1.2	0	1.1	0	0
6-mono-*O*-methyl (1,3,4,5-tetra-*O*-acetyl)	0	0	0.6	1.5	0
1,3,5-tri-*O*-methyl (4,6-di-*O*-acetyl)	0.9	0.7	0.8	0.7	0.9

[a] The solid line peak in Figure 4C. [b] Numbers in the table were calculated by making the italicized values as 1.0.

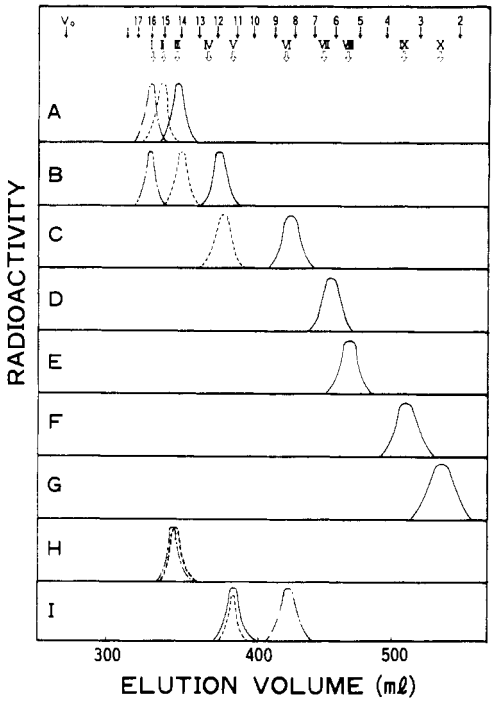


FIGURE 4: Sequential exoglycosidase digestion of oligosaccharides N4-1b, N4-2, and N4-3. Black arrows are the same as in Figure 3. White arrows indicate the eluting positions of authentic oligosaccharides: I, Gal_2·Fuc_2·$GlcNAc_2$·Man_3·GlcNAc·Fuc·$GlcNAc_{OT}$; II, Gal_2·Fuc·$GlcNAc_2$·Man_3·GlcNAc·Fuc·$GlcNAc_{OT}$; III, Gal_2·$GlcNAc_2$·Man_3·GlcNAc·Fuc·$GlcNAc_{OT}$; IV, $GlcNAc_2$·Man_3·GlcNAc·Fuc·$GlcNAc_{OT}$; V, Gal·GlcNAc·Man_3·GlcNAc·Fuc·$GlcNAc_{OT}$; VI, Man_3·GlcNAc·Fuc·$GlcNAc_{OT}$; VII, Man·GlcNAc·Fuc·$GlcNAc_{OT}$; VIII, GlcNAc·Fuc·$GlcNAc_{OT}$; IX, Fuc·$GlcNAc_{OT}$; X, *N*-acetyl[^{3}H]glucosaminitol. (A) Oligosaccharides N4-1b (—), N4-2 (---), and N4-3 (···); (B) oligosaccharides N4-1b (—) N4-2 (---), and N4-3 (···) incubated with jack bean β-galactosidase; (C) oligosaccharides in (B) derived from N4-1b (—) and N4-2 (---) incubated with jack bean β-*N*-acetylhexosaminidase; (D) oligosaccharide in (C) derived from N4-1b incubated with jack bean α-mannosidase; (E) the radioactive peak in (D) incubated with β-mannosidase; (F) the radioactive peak in (E) incubated with jack bean β-*N*-acetylhexosaminidase; (G) the radioactive peak in (F) incubated with *Charonia lampas* α-fucosidase; (H) oligosaccharides N4-2 (---) and N4-3 (···) incubated with almond α-fucosidase I; (I) oligosaccharides in (H) derived from N4-2 (---) and N4-3 (···) and intact N4-1b (—) incubated with a mixture of diplococcal β-galactosidase and diplococcal β-*N*-acetylhexosaminidase.

both oligosaccharides were slightly different. No degradation occurred when N4-2 and N4-3 were incubated with *Bacillus* α-fucosidase. These results indicated that N4-2 and N4-3 have one and two Galβ1→(3- or -4)[Fucα1→(4- or -3)]GlcNAc groups in their outer chain moieties, respectively. Both radioactive products in Figure 4H gave exactly the same degradation pattern as N4-1b (Figure 4β–G) by sequential exoglycosidase digestion. Therefore these oligosaccharides should also have the structure Galβ1→GlcNAcβ1→Manα1→6-(Galβ1→GlcNAcβ1→Manα1→3)Manβ1→4GlcNAcβ1→4-(Fucα1→6)$GlcNAc_{OT}$. The structural differences included in these three oligosaccharides were revealed by the following experiments.

When incubated with a mixture of diplococcal β-galactosidase and diplococcal β-*N*-acetylhexosaminidase, the defucosyl N4-3 was converted to Man_3·GlcNAc·Fuc·$GlcNAc_{OT}$, but N4-1b and the defucosyl N4-2 were converted to a radioactive component with the same mobility as authentic Gal·GlcNAc·Man_3·GlcNAc·Fuc·$GlcNAc_{OT}$ (Figure 4I). These results indicated that the two outer chains of defucosyl N4-3 are Galβ1→4GlcNAc while one of the two outer chains of N4-1b and defucosyl N4-2 are either Galβ1→3GlcNAc or Galβ1→6GlcNAc. The methylation analysis in Table I indicated that both N4-1b and N4-2 have 1 mol of C-3 substituted *N*-acetylglucosamine but not C-6-substituted *N*-acetylglucosamine. Therefore, these oligosaccharides should have one each of Galβ1→4GlcNAc and Galβ1→3GlcNAc groups in their outer chain moieties. The data in Table I also indicate that both N4-2 and N4-3 have all of their galactose and fucose residues as nonreducing termini and N4-1b, N4-2, and N4-3 have one, two, and three fucoses, respectively. Detection of 6-mono-*O*-methyl-2-(*N*-methylacetamido)-2-deoxyglucitol in both N4-2 and N4-3 indicates that the fucose residues in the outer chain moieties of these oligosaccharides occur as Galβ1→4(Fucα1→3)GlcNAc groups. Detection of approximately 2 mol of 3,4,6-tri-*O*-methylmannitol in all three oligosaccharides indicates that their two outer chain moieties are linked at the C-2 positions of two α-mannosyl residues of the trimannosyl core. On the basis of the results so far described, the structures of N4-1b, N4-2, and N4-3 were proposed as shown in Figure 8. That the Galβ1→3GlcNAc groups in N4-1b and N4-2 are located on the Manα1→3 side was confirmed as follows. When the radioactive components shown in Figure 4I obtained from N4-1b and N4-2 were incubated with jack bean α-mannosidase, no mannose residue was removed from both oligosaccharides (data not shown). Since this enzyme releases a mannose residue from R→-Manα1→6(Manα1→3)Manβ1→4GlcNAc—but not from

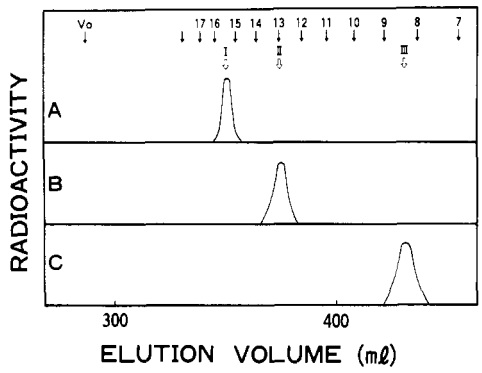
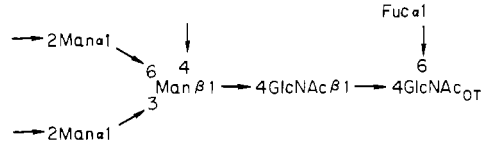


FIGURE 5: Sequential exoglycosidase digestion of oligosaccharide N4-1u. Black arrows are the same as in Figure 3. White arrows indicate the eluting positions of authentic oligosaccharides: I, Gal_2·$GlcNAc_2$·GlcNAc·Man_3·GlcNAc·Fuc·$GlcNAc_{OT}$; II, $GlcNAc_2$·GlcNAc·Man_3·GlcNAc·Fuc·$GlcNAc_{OT}$; III, Man_3·GlcNAc·Fuc·$GlcNAc_{OT}$. (A) Oligosaccharide N4-1u; (B) oligosaccharide N4-1u incubated with jack bean β-galactosidase; (C) the radioactive peak in (B) incubated with jack bean β-*N*-acetylhexosaminidase.

Manα1→6(R→Manα1→3)Manβ1→4GlcNAc—(Yamashita et al., 1980b), the Galβ1→3GlcNAcβ1→2 outer chains in the two radioactive oligosaccharides should be linked only to the Manα1→3 residue.

Upon Bio-Gel P-4 column chromatography, N4-1u was eluted as single peak with the same mobility as authentic Gal_2·$GlcNAc_2$·GlcNAc·Man_3·GlcNAc·Fuc·$GlcNAc_{OT}$ (Figure 5A). When incubated with jack bean β-galactosidase, it was converted to a radioactive component with the same mobility as authentic $GlcNAc_2$·GlcNAc·Man_3·GlcNAc·Fuc·$GlcNAc_{OT}$ (Figure 5B). It was then converted to a radioactive component with the same mobility as authentic Man_3·GlcNAc·Fuc·$GlcNAc_{OT}$ by jack bean β-*N*-acetylhexosaminidase digestion (Figure 5C). That this component actually has the structure $(Man\alpha1\rightarrow)_2Man\beta1\rightarrow GlcNAc\beta1\rightarrow(Fuc\alpha1\rightarrow)GlcNAc_{OT}$ was confirmed by further sequential exoglycosidase digestion, the data of which were completely the same as shown in Figure 4D–G. Methylation analysis of N4-1u in Table I indicates that the oligosaccharide has the following hexasaccharide as its core to which a β-*N*-acetylglucosamine residue and two Galβ1→4GlcNAcβ1→ groups are linked.



That the β-*N*-acetylglucosamine residue is exclusively linked to the C-4 position of the β-mannosyl residue was confirmed by periodate oxidation. When the periodate oxidation product of radioactive N4-1u was analyzed by Bio-Gel P-4 column, two radioactive components were detected (Figure 6A). The major component should be Manβ1→4GlcNAcβ1→4GlcNAc$_{OT}$ because it was converted to *N*-acetyl[^{3}H]-glucosaminitol by sequential digestion with β-mannosidase and jack bean β-*N*-acetylhexosaminidase (data not shown). The minor component is considered as a byproduct and not GlcNAcβ1→4Manβ1→4GlcNAcβ1→4GlcNAc$_{OT}$ because it was converted to a radioactive component which moved faster than Manβ1→4GlcNAcβ1→4GlcNAc$_{OT}$ by 2.0 glucose units in the Bio-Gel P-4 column after β-*N*-acetylhexosaminidase digestion. This minor component was also detected in the periodate oxidation product of authentic Gal_2·$GlcNAc_2$·Man_3·GlcNAc·Fuc·$GlcNAc_{OT}$ which does not contain the

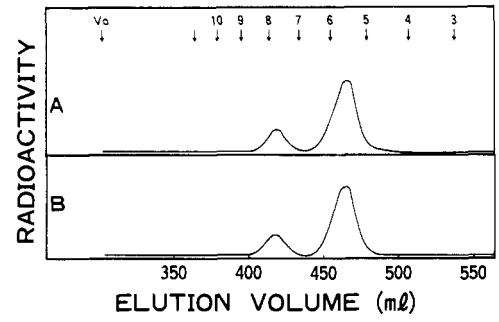


FIGURE 6: Periodate oxidation products of oligosaccharide N4-1u. For details of the periodate oxidation see Experimental Procedures. The black arrows are the same as in Figure 3. (A) The products from radioactive oligosaccharide N4-1u; (B) the products from authentic Gal_2·$GlcNAc_2$·Man_3·GlcNAc·Fuc·$GlcNAc_{OT}$.

GlcNAcβ1→4Manβ1→ group (Figure 6B). Therefore, the Galβ1→4GlcNAc group should not be linked at the C-4 position of the β-mannosyl residue because such a structure would produce the GlcNAcβ1→4Manβ1→ group after periodate oxidation. On the basis of the results so far described, the structure of N4-1u is proposed as shown in Figure 8.

Structures of N4-4, N4-5, N4-6, and N4-7 Fractions. Upon Bio-Gel P-4 column chromatography, N4-4, N4-5, and N4-6 were eluted as single peaks and the mobilities of N4-4 and N4-6 were the same as those of authentic Gal_3·$GlcNAc_3$·Man_3·GlcNAc·Fuc·$GlcNAc_{OT}$ and Gal_4·$GlcNAc_4$·Man_3·GlcNAc·Fuc·$GlcNAc_{OT}$, respectively (Figure 7A). In contrast, N4-7 was separated into two components as indicated by bars I and II in Figure 7A. These two components were collected separately and named as N4-7I and N4-7II, respectively.

When incubated with almond α-fucosidase I, N4-5 and N4-7I released 1 mol and N4-7II 2 mol of fucose residue (Figure 7B). The product from N4-5 gave exactly the same results as N4-4 and the products from N4-7I and N4-7II as N4-6 by the structural analyses described below.

When incubated with jack bean β-galactosidase, 3 and 4 mol of galactose were removed from N4-4 and N4-6 (Figure 7C). The products from N4-4 and N4-6 were then converted to a radioactive component with the same mobility as authentic Man_3·GlcNAc·Fuc·$GlcNAc_{OT}$ releasing 3 and 4 mol of *N*-acetylglucosamine by jack bean β-*N*-acetylhexosaminidase digestion, respectively (Figure 7D). That the products from N4-4 and N4-6 at this stage have the structure Manα1→6-(Manα1→3)Manβ1→4GlcNAcβ1→4(Fucα1→6)GlcNAc$_{OT}$ was confirmed as in the case of the solid line peak in Figure 4C. The sequential exoglycosidase digestion so far described indicates that the structure of N4-4 can be written as $(Gal\beta1\rightarrow GlcNAc\beta1\rightarrow)_3[Man\alpha1\rightarrow6(Man\alpha1\rightarrow3)Man\beta1\rightarrow$-4GlcNAcβ1→4(Fucα1→6)GlcNAc$_{OT}$] and that of N4-6 as $(Gal\beta1\rightarrow GlcNAc\beta1\rightarrow)_4[Man\alpha1\rightarrow6(Man\alpha1\rightarrow3)Man\beta1\rightarrow$-4GlcNAcβ1→4(Fucα1→6)GlcNAc$_{OT}$].

The methylation analysis of N4-4 in Table II indicated that a Galβ1→4GlcNAcβ1→ group is linked at the C-2 position of one α-mannosyl residue and two Galβ1→4GlcNAcβ1→ groups are linked at C-2 and C-4 positions of another α-mannosyl residue of the core portion. For determination of which of the two α-mannosyl residues is C-2,4 disubstituted, radioactive N4-4 was incubated with a mixture of diplococcal β-galactosidase and diplococcal β-*N*-acetylhexosaminidase. When the reaction mixture was analyzed by a Bio-Gel P-4 column, a single radioactive component with the same mobility as authentic GlcNAc·Man_3·GlcNAc·Fuc·$GlcNAc_{OT}$ was detected (Figure 7E). On the basis of the substrate specificity

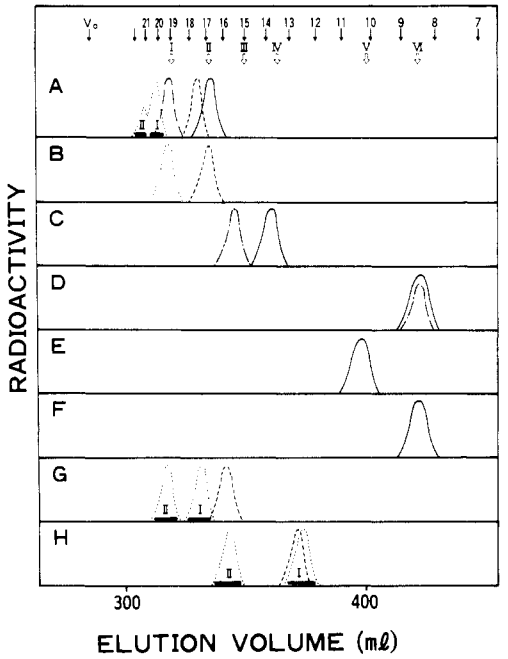


FIGURE 7: Sequential exoglycosidase digestion of oligosaccharides N4-4, N4-5, N4-6, and N4-7. Black arrows are the same as in Figure 3. White arrows indicate the eluting positions of authentic oligosaccharides: I, Gal_4·GlcNAc$_4$·Man_3·GlcNAc·Fuc·GlcNAc$_{OT}$; II, Gal_3·GlcNAc$_3$·Man_3·GlcNAc·Fuc·GlcNAc$_{OT}$; III, GlcNAc$_4$·Man_3·GlcNAc·Fuc·GlcNAc$_{OT}$; IV, GlcNAc$_3$·Man_3·GlcNAc·Fuc·GlcNAc$_{OT}$; V, GlcNAc·Man_3·GlcNAc·Fuc·GlcNAc$_{OT}$; VI, Man_3·GlcNAc·Fuc·GlcNAc$_{OT}$. (A) Oligosaccharides N4-4 (—), N4-5 (---), N4-6 (---), and N4-7 (···); (B) oligosaccharides N4-5 (---) N4-7I (···), and N4-7II (···) incubated with almond α-fucosidase; (C) oligosaccharides N4-4 (—) and N4-6 (---) incubated with jack bean β-galactosidase; (D) the radioactive peaks in (C) incubated with jack bean β-*N*-acetylhexosaminidase; (E) oligosaccharide N4-4 incubated with a mixture of diplococcal β-galactosidase and diplococcal β-*N*-acetylhexosaminidase; (F) the radioactive peak in (E) incubated with jack bean β-*N*-acetylhexosaminidase; (G) oligosaccharides N4-5 (---), N4-7I (···), and N4-7II (···) incubated with jack bean β-galactosidase; (H) the radioactive peaks in (G) incubated with jack bean β-*N*-acetylhexosaminidase.

of diplococcal β-*N*-acetylhexosaminidase (for details see Experimental Procedures), the structure of the radioactive component in Figure 7E was concluded to be GlcNAcβ1→4Manα1→(3- or -6)[Manα1→(6- or -3)]Manβ1→4GlcNAcβ1→4(Fucα1→6)GlcNAc$_{OT}$. Actually the radioactive component in Figure 7E was converted to Manα1→6(Manα1→3)Manβ1→4GlcNAcβ1→4(Fucα1→6)GlcNAc$_{OT}$ by jack bean β-*N*-acetylhexosaminidase digestion releasing 1 mol of *N*-acetylglucosamine (Figure 7F). When the radioactive component in Figure 7E was incubated with jack bean α-mannosidase, no degradation occurred (data not shown). This result indicated that an *N*-acetylglucosamine residue should be linked exclusively to the Manα1→3 side. Therefore, the structure of N4-4 is proposed as shown in Figure 8.

Methylation analysis of N4-6 indicated that two Galβ1→4GlcNAcβ1→ groups are linked at the C-2 and C-4 positions of one α-mannosyl residue and two Galβ1→4GlcNAcβ1→ groups at the C-2 and C-6 positions of the other α-mannosyl residue of the trimannosyl core as shown in Figure 8. The exact location of C-2,4 and C-2,6 disubstitution could not be determined because of the limited amount of sample available.

As already described, N4-5 is a monofucosyl derivative of N4-4, and the fucose residue should be linked at the C-3 position of the *N*-acetylglucosamine residue of one of the three Galβ1→4GlcNAcβ1→ outer chains of N4-4. When incubated sequentially with jack bean β-galactosidase and β-*N*-acetylhexosaminidase, 2 mol each of galactose and *N*-acetylglucosamine was removed from N4-5 [Figure 7G,H (---)], respectively. The radioactive product in Figure 7H (---) was completely resistant to jack bean α-mannosidase digestion, indicating that the remaining Galβ1→4(Fucα1→3)GlcNAcβ1→ outer chain is linked only to Manα1→3 side. A fucose residue and a galactose residue were removed from the product in Figure 7H by sequential digestion with almond α-fucosidase I and jack bean β-galactosidase (data not shown). The resulting product was completely resistant to diplococcal β-*N*-acetylhexosaminidase treatment. These results indicated that the *N*-acetylglucosamine residue of the Galβ1→4(Fucα1→3)GlcNAcβ1→ group should exclusively be linked at the C-4 position of the Manα1→3 residue. On the basis of a series of results described above, the structure of N4-5 is proposed as shown in Figure 8.

Table II: Methylation Analysis of N4-4, N4-5, N4-6, and N4-7

partially methylated sugars	molar ratio[a] N4-4	N4-5	N4-6	N4-7
fucitol				
2,3,4-tri-*O*-methyl (1,5-di-*O*-acetyl)	0.9	2.2	1.1	2.5
galactitol				
2,3,4,6-tetra-*O*-methyl (1,5-di-*O*-acetyl)	3.2	3.5	4.4	4.5
mannitol				
3,4,6-tri-*O*-methyl (1,2,5-tri-*O*-acetyl)	1.2	1.4	0	0
3,6-di-*O*-methyl (1,2,4,5-tetra-*O*-acetyl)	0.7	0.7	0.8	0.9
3,4-di-*O*-methyl (1,2,5,6-tetra-*O*-acetyl)	0	0	0.6	0.6
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				
3,6-di-*O*-methyl (1,4,5-tri-*O*-acetyl)	4.4	3.5	5.3	3.7
6-mono-*O*-methyl (1,3,4,5-tetra-*O*-acetyl)	0	0.6	0	1.3
1,3,5-tri-*O*-methyl (4,6-di-*O*-acetyl)	1.3	1.2	1.3	1.3

[a] Numbers in the table were calculated by making the italicized values as 1.0.

As already described, N4-7I and N4-7II were mono- and difucosyl derivatives of N4-6. Because the fucosyl residues could be removed by almond α-fucosidase I, they should occur as the Galβ1→4(Fucα1→3)GlcNAcβ1→ group. Detection of 6-mono-*O*-methyl-2-(*N*-methylacetamido)-2-deoxyglucitol in the methylation data of the N4-7 fraction (Table II) supports this assumption.

When incubated sequentially with jack bean β-galactosidase and jack bean β-*N*-acetylhexosaminidase, 3 mol each of galactose and *N*-acetylglucosamine was removed from N4-7I and 2 mol each of galactose and *N*-acetylglucosamine from N4-7II [Figure 7G,H (···)]. The radioactive products in Figure 7H from N4-7I and N4-7II were completely resistant to jack bean α-mannosidase digestion. Therefore, the Galβ1→4(Fucα1→3)GlcNAcβ1→ group of N4-7I and at least one of the two Galβ1→4(Fucα1→3)GlcNAcβ1→ groups of N4-7II should be linked to the Manα1→3 side of the trimannosyl core. A fucose and a galactose were removed from peak I in Figure 7H by sequential digestion with almond α-fucosidase I and jack bean β-galactosidase (data not shown). The radioactive heptaitol thus obtained was converted to Manα1→6(Manα1→3)Manβ1→4GlcNAcβ1→4(Fucα1→6)GlcNAc$_{OT}$ by jack bean β-*N*-acetylhexosaminidase digestion but was

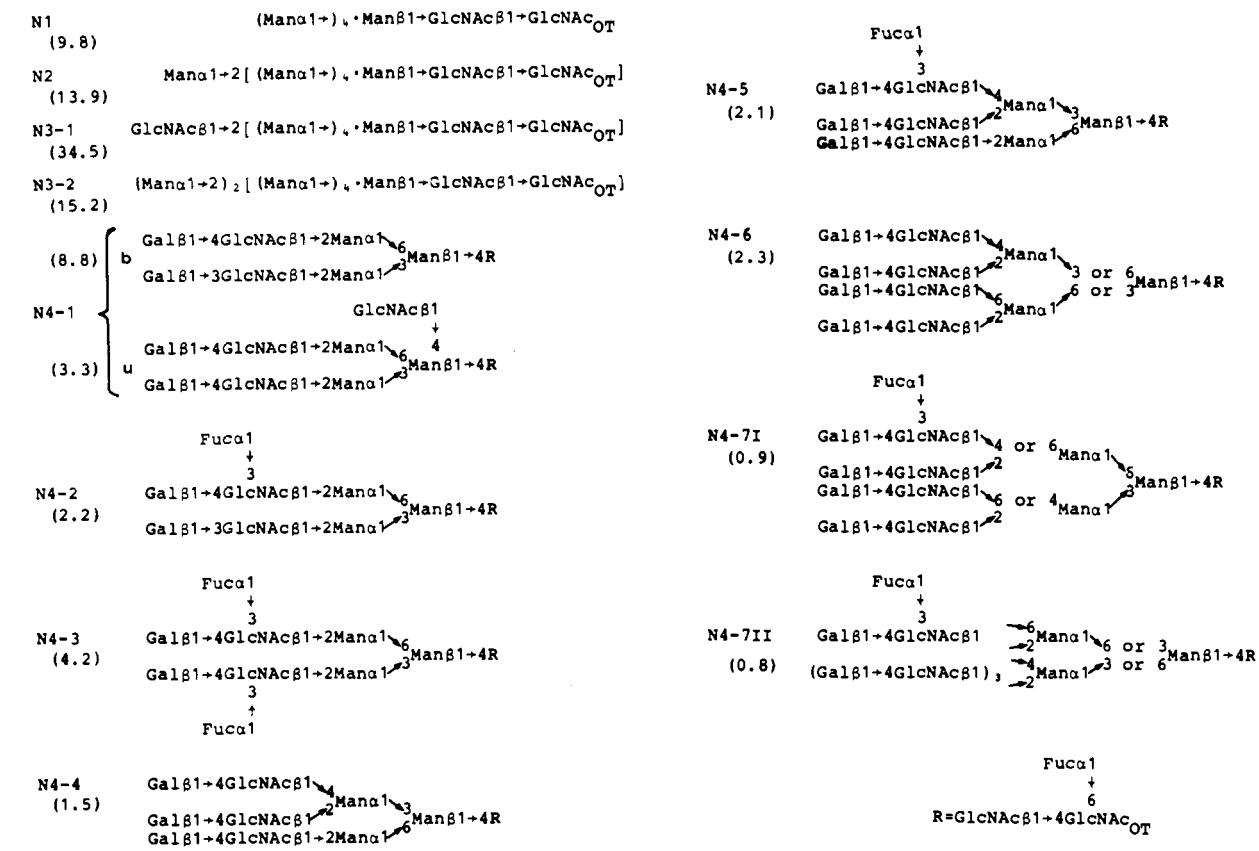


FIGURE 8: Structures of neutral oligosaccharides obtained from X-31 BHA glycoprotein. Numbers in parentheses are percentage molar ratio of the sugar chains in BHA.

totally resistant to diplococcal β-*N*-acetylhexosaminidase treatment (data not shown). These results indicate that the Galβ1→4(Fucα1→3)GlcNAcβ1→ group of N4-7I is linked to either the C-4 or C-6 position of α-mannosyl residue as shown in Figure 8.

So far, the exact location of the two Galβ1→4(Fucα1→-3)GlcNAcβ1→ groups in N4-7II has not been determined.

Discussion

The complete amino acid sequence of the HA of X-31 strain of A2/Hong Kong/1968 virus was elucidated by Ward & Dopheide (1981) and by Verhoeyen et al. (1980). The data indicated that the HA contains seven asparagine-linked sugar chains. These sugar chains are attached at asparagine residues 8, 22, 38, 81, 165, and 285 in the HA1 subunit and 154 in the HA2 subunit, and the sugar chain on asparagine-8 of HA1 subunit is supposed to contain a sulfate residue. Several works concerning the carbohydrate moieties of HA have been reported (Schwarz & Klenk, 1981; Ward & Dopheide, 1981). However, the information reported in these papers was limited because it was based either on the monosaccharide compositions of glycopeptides obtained by cyanogen bromide fragmentation or on gel permeation analysis of the glycopeptides obtained by exhaustive Pronase digestion of the metabolically labeled HA with radioactive monosaccharides.

Because BHA contains most of the sugar chains of intact HA, the results reported in this paper may represent most of the characteristics of the sugar chains of HA. Approximately 20% of the sugar chains of BHA was acidic. This result indicates that more than a single sugar chain in BHA might be sulfated. However, this conclusion must be refrained until more structural information on the acidic sugar chains will be obtained, because the action of sulfatase on the acidic sugar chains was far from quantitative. The structures of almost all of the neutral sugar chains of BHA were elucidated in this paper. The results summarized in Figure 8 indicate that the sugar chains of influenza virus hemagglutinin are formed by the processing pathway (Kornfeld & Kornfeld, 1980) because N1, N2, N3-1, and N3-2 accord with the processing intermediates. Structures of the remaining oligosaccharides, however, indicate that these sugar chains are formed by complicated routes. N4-1b and N4-2 should be formed by the pathway different from that forming other sugar chains. In addition, N4-1u, N4-3, N4-5, and N4-7II are considered as final products in their biosynthetic route. It is particularly noteworthy that only N4-1u among the complex type sugar chains contains the *N*-acetylglucosamine residue linked to β-mannose. In other glycoproteins which have this bisectional *N*-acetylglucosamine in their sugar chains, the *N*-acetylglucosamine residue is more widely distributed in their sugar chains. The oligosaccharide pattern of BHA reported here is somewhat different from those of HANA protein and F protein, the two envelope glycoproteins of HVJ which were also grown in the allantoic sac of embryonated hen eggs (Yoshima et al., 1981). The two glycoproteins of HVJ do not contain any tri- and tetraantennary complex type sugar chains. The complex type sugar chains with the Galβ1→3GlcNAc group in their outer chain moieties (N4-1b and N4-2 in Figure 8) and with an *N*-acetylglucosamine residue linked to β-mannose (N4-1u in Figure 8) are also absent in the two glycoproteins of HVJ. These differences may indicate that the sugar chains of envelope glycoproteins of the two viruses are not simple replicas of host membrane glycoproteins as suggested in the case of vesicular stomatitis virus (Hunt, 1980).

In Figure 8, the percent molar ratio of each sugar chain in the BHA molecule is also included. One of the prominent features is that the sum total of the complex type sugar chains is only 26.6% of all neutral sugar chains. Therefore the

complex type sugar chains can occupy at most two asparagine residues if they are attached at specific asparagine residues as suggested by several investigators. This estimation does not accord with the analytical result reported by Ward & Dopheide (1981) which showed that five asparagine loci of X-31 influenza hemagglutintin are occupied by complex type sugar chains. A possible explanation for this discrepancy is that many asparagine loci are substituted by both the complex type and the high mannose type sugar chains. The glycopeptide fragments containing such loci might have been concluded to have complex type based only on their monosaccharide compositions.

The possibility that the sugar chains were modified during bromelain treatment of HA to obtain BHA is rather improbable but cannot be completely denied at the present stage. Therefore, a comparative study of the oligosaccharide patterns of glycopeptide fragments with a single asparagine-linked sugar chain from HA is essential to confirm the above estimation.

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Registry No. N1, 83816-30-2; N2, 83816-31-3; N3-1, 83844-72-8; N3-2, 83830-93-7; N4-1 b, 83830-94-8; N4-1 u, 83816-32-4; N4-2, 83816-33-5; N4-3, 83368-45-0; N4-4, 80968-39-4; N4-5, 83830-95-9.

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