White, M. F., Takayama, S., & Kahn, R. C. (1985) *J. Biol. Chem. 260*, 9470-9478.
Zetterquist, O., Ragnarsson, U., Humble, E., Berglund, L., & Engstrom, L. (1976) *Biochem. Biophys. Res. Commun. 70*, 696-703.
Zhou, J., Trimmer, J. S., Agnew, W. S., Boyle, M. B., Cooperman, S. S., Goodman, R. H., Mandel, G., & Sigworth, F. J. (1989) *Biophys. J. 55*, 230a.

Diversity of Oligosaccharide Structures Linked to Asparagines of the Scrapie Prion Protein†

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ABSTRACT: Prion proteins from humans and rodents contain two consensus sites for asparagine-linked glycosylation near their C-termini. The asparagine-linked oligosaccharides of the scrapie isoform of the hamster prion protein (PrP 27-30) were released quantitatively from the purified molecule by hydrazinolysis followed by N-acetylation and NaB^3H_4 reduction. The radioactive oligosaccharides were fractionated into one neutral and three acidic oligosaccharide fractions by anion-exchange column chromatography. All oligosaccharides in the acidic fractions could be converted to neutral oligosaccharides by sialidase digestion. Structural studies on these oligosaccharides including sequential exoglycosidase digestion in combination with methylation analysis revealed that PrP 27-30 contains a mixture of bi-, tri-, and tetraantennary complex-type sugar chains with Manα1→6(GlcNAcβ1→4)(Manα1→3)Manβ1→4GlcNAcβ1→4-(Fucα1→6)GlcNAc as their core. Variation is produced by the different combination of the oligosaccharides Galβ1→4GlcNAcβ1→, Galβ1→4(Fucα1→3)GlcNAcβ1→, GlcNAcβ1→, Siaα2→3Galβ1→4GlcNAcβ1→, and Siaα2→6Galβ1→4GlcNAcβ1→ in their outer chain moieties. When both asparagine-linked consensus sites are glycosylated, the diversity of oligosaccharide structures yields over 400 different forms of the scrapie prion protein. Whether these diverse asparagine-linked oligosaccharides participate in scrapie prion infectivity or modify the function of the cellular prion protein remains to be established.

Scrapie is a degenerative neurological disease of sheep and goats, which can be transmitted to laboratory rodents (Prusiner, 1987). Because the unusual properties of the scrapie agent distinguish it from viruses, the term prion was introduced (Prusiner, 1982). Three human diseases may also be caused by prions: Creutzfeldt-Jakob disease, kuru, and Gerstmann-Sträussler syndrome (Gajdusek, 1977; Masters et al., 1981). Purification of scrapie prion infectivity led to isolation of a protein with relative molecular weight of 27000-30000 (Bolton et al., 1982; Prusiner et al., 1982). This protein was designated prion protein (PrP) 27-30.[1]

PrP cDNA clones were isolated from libraries that were constructed by using poly(A+)RNA from scrapie-infected brains of hamsters and mice (Chesebro et al., 1985; Oesch et al., 1985). Southern blotting revealed that PrP 27-30 is encoded by a cellular gene and not by the infectious prion particles (Oesch et al., 1985). Unexpectedly, Northern blotting indicated that normal and scrapie-infected brains contain similar levels of PrP mRNA (Chesebro et al., 1985; Oesch et al., 1985). The presence of PrP mRNA in normal brain led to the discovery of a normal cellular isoform of PrP, termed PrP^C, and the demonstration that PrP 27-30 is derived by limited proteolysis from a larger protein, designated PrP^{Sc} (Oesch et al., 1985). Sensitivity to proteinase K and solubility after detergent extraction differentiate PrP^{Sc} from PrP^C (Oesch et al., 1985; Meyer et al., 1986). Because the entire PrP open reading frame (ORF) is contained within a single exon, the differences between PrP^C and PrP^{Sc} are not due to alternative splicing of exons but probably result from a posttranslational event (Basler et al., 1986). To date, there is evidence for at least six posttranslational modifications of both PrP isoforms including glycosylation (Prusiner, 1987; Stahl et al., 1987; Turk et al., 1988; Haraguchi et al., unpublished observations). Since earlier studies suggested that PrP 27-30 is a sialoglycoprotein and the amino acid sequence of PrP contains two potential asparagine-linked glycosylation sites (Bolton et al., 1985; Oesch et al., 1985), we undertook a structural study of the sugar chains of PrP 27-30 with a view toward eventually determining whether the different properties of two PrP isoforms arise from differences in their asparagine-linked oligosaccharides. Recently, we demonstrated the presence of asparagine-linked oligosaccharides in both PrP isoforms, which were resistant to endoglycosidase H digestion but sensitive to *N*-glycanase

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[1] Abbreviations: PrP, prion protein; PrP^{Sc}, scrapie isoform of the prion protein; PrP^C, cellular isoform of the prion protein; PrP 27-30, scrapie prion protein derived from PrP^{Sc} by limited proteolysis. Subscript OT is used in this paper to indicate NaB^3H_4-reduced sugars. All sugars mentioned in this paper were of the D configuration, except for fucose, which had an L configuration.

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digestion (Haraguchi et al., unpublished observations).

In this paper, we report a detailed structural study of the sugar chains of PrP 27–30.

Experimental Procedures

Chemicals and Enzymes. NaB^3H_4 (348 mCi/mmol) was purchased from New England Nuclear, Boston, MA, and NaB^2H_4 (98%) from Merck Co., Darmstadt, FRG. *Arthrobacter ureafaciens* sialidase was purchased from Nacalai Tesque Inc., Kyoto. β-Galactosidase and β-*N*-acetylhexosaminidase were purified from the culture fluid of *Diplococcus pneumoniae* by the method of Glasgow et al. (1977). Another β-galactosidase, β-*N*-acetylhexosaminidase, and α-mannosidase were purified from jack bean meal according to the method of Li and Li (1972). Snail β-mannosidase was kindly supplied by Seikagaku Kogyo Co., Tokyo. Bovine epididymal α-fucosidase was purchased from Sigma Chemical Co., St. Louis, MO. α-Fucosidase I was purified from almond emulsin, and *Aspergillus saitoi* α-mannosidase II was purified as reported previously (Kobata, 1982; Amano & Kobata, 1986). *Datura stramonium* agglutinin (DSA)–Sepharose was prepared as described previously (Yamashita et al., 1987). *Ricinus communis* agglutinin (RCA) 120-WG003 was purchased from Hohnen Oil Co., Tokyo.

Scrapie Prions. Weanling random-bred (LVG/LAK) Syrian golden hamsters were inoculated intracerebrally with 10^7 ID_{50} units of Syrian hamster adapted scrapie prions in their fifth passage. These animals developed clinical signs of scrapie approximately 70 days after inoculation and were sacrificed 5 days later. Their brains were removed immediately upon sacrifice, frozen in liquid nitrogen, and stored at −70 °C.

Purification of PrP 27–30. Large-scale purification of PrP 27–30 from scrapie-infected hamster brains was accomplished with a series of detergent extractions, enzymatic digestions, differential centrifugations, and discontinuous sucrose gradient sedimentation as previously described (Prusiner et al., 1983). The purified PrP 27–30 is aggregated into rod-shaped structures that were dissociated by boiling the purified samples in 2% SDS for 2 min. PrP 27–30 was purified further by preparative SDS–PAGE. The buffer system of Laemmli (1970) was employed by using a 16 × 18 cm slab with a thickness of 0.75 mm composed of 15% polyacrylamide. A strip 1 cm wide was cut from the slab and the PrP 27–30 eluted into 10 mM NH_4HCO_3 buffer containing 0.1% SDS. The SDS was removed by repeated acetone precipitation (×6) and the sample dried by lyophilization.

Analytical Studies of PrP 27–30. The amount of purified PrP 27–30 was estimated by three independent methods: (a) silver staining of SDS–PAGE (Merril et al., 1981); (b) bicinchoninic acid (BCA) protein assay (Smith et al., 1985); and (c) amino acid analysis (Jones, 1986). The intensity of PrP 27–30 staining with silver was compared to that of known standard proteins. Protein determinations using the BCA reagent purchased from Pierce were performed by using crystalline bovine serum albumin fraction V purchased from Pentex as a standard. Amino acid analysis of hydrolyzed PrP 27–30 was accomplished by derivatization with fluoraldehyde–*o*-phthalaldehyde (OPA) purchased from Pierce. The mixture was separated on a C_{18} column by reverse-phase HPLC and the amount of protein estimated from the values obtained for the following amino acids: Asp, Glu, Ser, His, Thy, and Ile.

Glycosidase Digestion. Radioactive oligosaccharides (1–100 × 10^4 cpm) were incubated with one of the following mixtures at 37 °C for 24–48 h: *sialidase digestion*, 50 milliunits of enzyme in 0.1 M sodium acetate buffer, pH 5.0 (50 μL); *jack bean β-N-acetylhexosaminidase digestion*, 2 units of enzyme and 2.5 mg of galactono-1,5-lactone in 0.1 M sodium citrate buffer, pH 5.0 (50 μL); *digestion with a mixture of jack bean β-galactosidase and β-N-acetylhexosaminidase*, 0.6 unit of β-galactosidase and 5 units of β-*N*-acetylhexosaminidase in 0.1 M sodium citrate buffer, pH 4.0 (50 μL); *jack bean α-mannosidase digestion*, 0.5 unit of enzyme in 0.05 M sodium acetate buffer, pH 4.5, containing 1 mM $ZnCl_2$ (50 μL); *A. saitoi α-mannosidase II digestion*, 100 microunits of enzyme in 0.1 M acetate buffer, pH 5.0, containing 1 mM $CaCl_2$ (50 μL); *snail β-mannosidase digestion*, 10 milliunits of enzyme in 0.05 M sodium citrate buffer, pH 4.0 (50 μL); *epididymal α-fucosidase digestion*, 10 milliunits of enzyme in 0.05 M sodium citrate buffer, pH 6.0 (40 μL); *diplococcal β-galactosidase digestion*, 2 milliunits of enzyme in 0.1 M sodium citrate buffer, pH 6.0 (50 μL); *diplococcal β-N-acetylhexosaminidase digestion*, 10 milliunits of enzyme in 0.1 M sodium citrate buffer, pH 6.0 (50 μL); *almond α-fucosidase I digestion*, 40 microunits of enzyme in 0.15 M citrate phosphate buffer, pH 5.0 (30 μL). A small amount of toluene was added to all reaction mixtures to inhibit bacterial growth during incubation.

Oligosaccharides. Neu5Acα2→6Galβ1→4GlcNAcβ1→2Manα1→6[Neu5Acα2→3Galβ1→4GlcNAcβ1→4(Neu5Acα2→6Galβ1→4GlcNAcβ1→2)Manα1→3]Manβ1→4GlcNAcβ1→4GlcNAc$_{OT}$ (Neu5Ac$_3$·Gal$_3$·GlcNAc$_3$·Man$_3$·GlcNAc·GlcNAc$_{OT}$), Neu5Acα2→6Galβ1→4GlcNAcβ1→2Manα1→6(Neu5Acα2→6Galβ1→4GlcNAcβ1→2Manα1→3)Manβ1→4GlcNAcβ1→4GlcNAc$_{OT}$ (Neu5Ac$_2$·Gal$_2$·GlcNAc$_2$·Man$_3$·GlcNAc·GlcNAc$_{OT}$), and Neu5Acα2→6Galβ1→4GlcNAcβ1→2Manα1→6(Galβ1→4GlcNAcβ1→2Manα1→3)Manβ1→4GlcNAcβ1→4GlcNAc$_{OT}$ (Neu5Ac·Gal$_2$·GlcNAc$_2$·Man$_3$·GlcNAc·GlcNAc$_{OT}$) were prepared from human ceruloplasmin by hydrazinolysis followed by reduction with NaB^3H_4 (Yamashita et al., 1981). Galβ1→4GlcNAcβ1→2Manα1→6(GlcNAcβ1→4)(Galβ1→4GlcNAcβ1→2Manα1→3)Manβ1→4GlcNAcβ1→4(Fucα1→6)GlcNAc$_{OT}$ (Gal$_2$·GlcNAc$_2$·Man$_2$·GlcNAc·Man·GlcNAc·Fuc·GlcNAc$_{OT}$), Galβ1→4GlcNAcβ1→2Manα1→6(GlcNAcβ1→4)(GlcNAcβ1→2Manα1→3)Manβ1→4GlcNAcβ1→4(Fucα1→6)GlcNAc$_{OT}$ (Gal·GlcNAc$_2$·Man$_2$·GlcNAc·Man·GlcNAc·Fuc·GlcNAc$_{OT}$), and GlcNAcβ1→2Manα1→6(GlcNAcβ1→4)(GlcNAcβ1→2Manα1→3)Manβ1→4GlcNAcβ1→4(Fucα1→6)GlcNAc$_{OT}$ (GlcNAc$_2$·Man$_2$·GlcNAc·Man·GlcNAc·Fuc·GlcNAc$_{OT}$) were obtained from human immunoglobulin G (Mizuochi et al., 1982). Manα1→6(Manα1→3)Manβ1→4GlcNAcβ1→4(Fucα1→6)GlcNAc$_{OT}$ (Man$_3$·GlcNAc·Fuc·GlcNAc$_{OT}$) was obtained by jack bean β-*N*-acetylhexosaminidase digestion of GlcNAc$_2$·Man$_2$·GlcNAc·Man·GlcNAc·Fuc·GlcNAc$_{OT}$. Manα1→6(Galβ1→4GlcNAcβ1→2Manα1→3)Manβ1→4GlcNAcβ1→4(Fucα1→6)GlcNAc$_{OT}$ (Gal·GlcNAc·Man$_3$·GlcNAc·Fuc·GlcNAc$_{OT}$) (Endo et al., 1987), [Galβ1→4(Fucα1→3)GlcNAcβ1→2Manα1→6](Manα1→3)Manβ1→4GlcNAcβ1→4(Fucα1→6)GlcNAc$_{OT}$ (Gal·Fuc·GlcNAc·Man$_3$·GlcNAc·Fuc·GlcNAc$_{OT}$) (Endo et al., 1986), GlcNAcβ1→6(GlcNAcβ1→2)Manα1→6(GlcNAcβ1→4)[GlcNAcβ1→4(GlcNAcβ1→2)Manα1→3]Manβ1→4GlcNAcβ1→4(Fucα1→6)GlcNAc$_{OT}$ (GlcNAc$_4$·Man$_2$·GlcNAc·Man·GlcNAc·Fuc·GlcNAc$_{OT}$), and GlcNAcβ1→2Manα1→6(GlcNAcβ1→4)[GlcNAcβ1→4(GlcNAcβ1→2)Manα1→3]Manβ1→4GlcNAcβ1→4(Fucα1→6)GlcNAc$_{OT}$ (GlcNAc$_3$·Man$_2$·GlcNAc·Man·GlcNAc·Fuc·GlcNAc$_{OT}$) (Yamashita et al., 1983) were obtained according to the

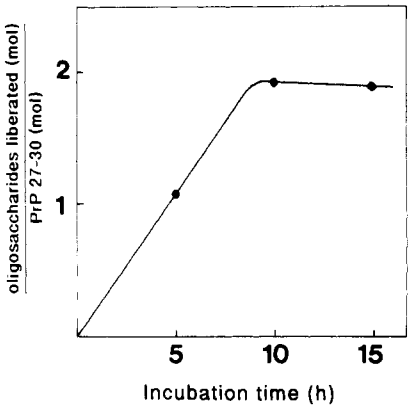


FIGURE 1: Time course of the release of the asparagine-linked oligosaccharides from PrP 27–30 by hydrazinolysis.

methods of the cited references.

Analytical Methods. Anion-exchange chromatography was carried out on a FPLC apparatus (Pharmacia LKB Biotechnology Inc., Tokyo) equipped with a Mono Q HR5/5 column. The elution was performed with 5 mM sodium acetate, pH 4.0, for 10 min, then with a linear gradient from 5 to 300 mM sodium acetate (pH 4.0) for 30 min, and finally with a linear gradient from 300 to 500 mM sodium acetate (pH 4.0) for 10 min. Chromatography was performed at room temperature at a flow rate of 1 mL/min. Oligosaccharides were fractionated on columns of Bio-Gel P-4 at 55 °C with a mixture of glucose oligomers as internal standards (Yamashita et al., 1982). Methylation analysis of oligosaccharides was carried out as described earlier (Endo et al., 1979) by using a JEOL DX-300 gas chromatograph–mass spectrometer (JEOL, Ltd., Tokyo), the capillary column (0.25 mm × 30 m) of DB5-30N (J&W Scientific Inc., Cordova, CA), and the column temperature programmed at 8 °C increments/min from 95 to 280 °C. Lectin affinity chromatography on a column of RCA 120-WG003 and DSA were performed as described previously (Harada et al., 1987; Yamashita et al., 1987).

Results

PrP 27–30 Contains Two Asparagine-Linked Oligosaccharides. The hamster PrP gene encodes a protein of 254 amino acids with two consensus sites for asparagine-linked glycosylation at codons 181 and 197. To determine the number of asparagine-linked oligosaccharides attached to PrP 27–30, 26 nmol of PrP 27–30 was subjected to hydrazinolysis for varying periods of time (Takasaki et al., 1982). As shown in Figure 1, 10 h of hydrazinolysis is sufficient to liberate all asparagine-linked oligosaccharides from PrP 27–30, and approximately 2 mol of oligosaccharides was liberated from 1 mol of PrP 27–30 by hydrazinolysis. On the basis of these results, 2.5 mg of PrP 27–30 was subjected to hydrazinolysis for 10 h. One-third of the oligosaccharide fraction was reduced with NaB^3H_4, and the remainder was reduced with 2 mg of NaB^2H_4 to obtained deuterium-labeled oligosaccharide fraction for methylation analysis.

Sialylated Oligosaccharides from PrP 27–30. The radioactive oligosaccharide mixture obtained from PrP 27–30 was subjected to anion-exchange column chromatography with the Mono Q HR5/5 column. As shown in Figure 2A, one neutral (N) and three acidic (A1–3) fractions were obtained. When a mixture of A1, A2, and A3 was digested exhaustively with sialidase, it was completely converted to neutral oligosaccharides (AN in Figure 2B). Mild acid hydrolysis (0.01 N HCl at 100 °C for 3 min) of A2 and A3, after which part of the original acidic component still remained, gave one and

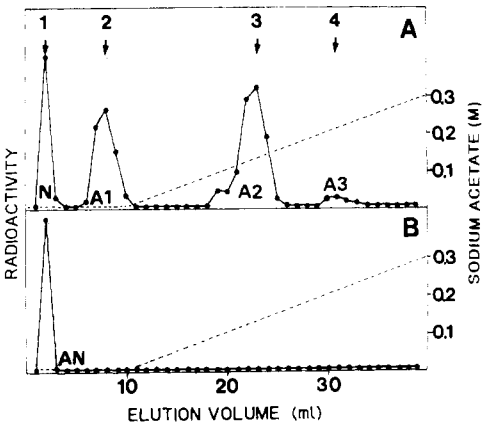


FIGURE 2: Anion-exchange chromatography of oligosaccharides released from PrP 27–30 (A) and of sialidase digests of the acidic oligosaccharides (pooled sample of A1–3) (B). The arrows indicate the positions where authentic oligosaccharides were eluted: 1, Gal_2·$GlcNAc_2$·Man_2·GlcNAc·Man·GlcNAc·Fuc·$GlcNAc_{OT}$; 2, Neu5Ac·Gal_2·$GlcNAc_2$·Man_3·GlcNAc·$GlcNAc_{OT}$; 3, $Neu5Ac_2$·Gal_2·$GlcNAc_2$·Man_3·GlcNAc·$GlcNAc_{OT}$; 4, $Neu5Ac_3$·Gal_3·$GlcNAc_3$·Man_3·GlcNAc·$GlcNAc_{OT}$.

two additional acidic components, respectively, with a neutral component (data not shown). In contrast, fraction A1, treated under the same condition, gave only a neutral product (data not shown). These results indicated that A1, A2, and A3 contain one, two, and three sialic acid residues, respectively. The percent molar ratio of neutral, monosialylated, disialylated, and trisialylated oligosaccharide fractions was 27:20:48:5 on the basis of their radioactivities.

Methylation Analysis of Oligosaccharides. Since it was not possible to obtain interpretable methylation data for individual oligosaccharides, the elucidation of oligosaccharide structure was based mainly on sequential exoglycosidase digestion and lectin affinity chromatography. However, for interpretation of the results, knowledge of the sugar linkages present in the oligosaccharides was required and, thus, fractions N, AN, and A (a pool of A1–3) were subjected to methylation analysis (Table I). The data indicated that all oligosaccharides in these fractions had bisecting *N*-acetylglucosamine residues because 2-mono-*O*-methylmannitol but not 2,4-di-*O*-methylmannitol was detected. Therefore, the relative amounts of each monosaccharide fragment were calculated by taking the value of 2-mono-*O*-methylmannitol as 1.0.

Detection of 1,3,5-tri- but not 1,3,5,6-tetra-*O*-methyl-2-(*N*-methylacetamido)-2-deoxyglucitol indicated that *N*-acetylglucosamine residues at the reducing termini of all oligosaccharides are substituted both at the C-4 and at the C-6 positions. From the results of glycosidase digestion, the C-6 position of this *N*-acetylglucosamine residue should be occupied by an α-fucosyl residue. Galactose residues of the oligosaccharides in fraction AN occur as nonreducing termini because only 2,3,4,6-tetra-*O*-methylgalactitol was detected. Detection of 6-mono-*O*-methyl 2-(*N*-methylacetamido)-2-deoxyglucitol in fraction AN indicated that either Le^a or X-antigenic determinant is included in the outer chain moieties of many oligosaccharides. Detection of more than 1 mol of 3,4,6-tri-*O*-methyl-2-(*N*-methylacetamido)-2-deoxyglucitol in fraction N indicated that some of the oligosaccharides in this fraction contained a nonreducing terminal *N*-acetylglucosamine residue in their outer chain moieties.

Comparison of the data obtained by methylation analysis of fractions AN and A indicated that difference was found only in the galactitol derivatives. The decrease of the 2,3,4,6-tetra-*O*-methylgalactitol in fraction A was balanced by the presence of 2,3,4- and 2,4,6-tri-*O*-methylgalactitols.

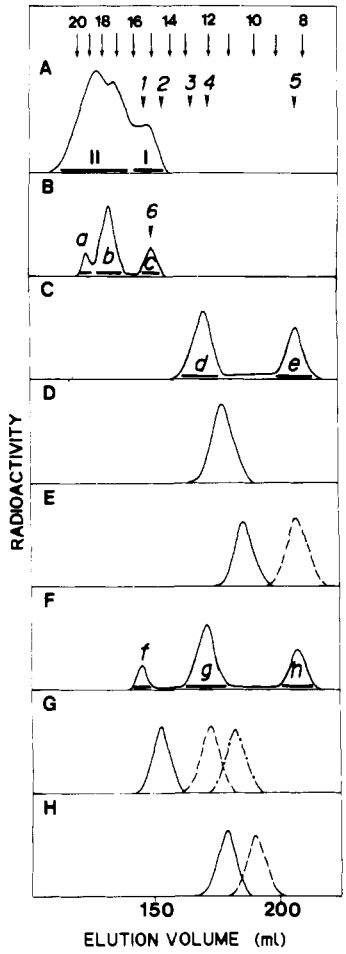


FIGURE 3: Bio-Gel P-4 column chromatography of oligosaccharide fraction AN and its sequential exoglycosidase digestion products. Arrows at the top indicate the elution positions of glucose oligomers (the numbers indicate the glucose units). Arrow heads indicate the elution positions of authentic oligosaccharides: **1**, $GlcNAc_4 \cdot Man_2 \cdot GlcNAc \cdot Man \cdot GlcNAc \cdot Fuc \cdot GlcNAc_{OT}$; **2**, $GlcNAc_3 \cdot Man_2 \cdot GlcNAc \cdot Man \cdot GlcNAc \cdot Fuc \cdot GlcNAc_{OT}$; **3**, $GlcNAc_2 \cdot Man_2 \cdot GlcNAc \cdot Man \cdot GlcNAc \cdot Fuc \cdot GlcNAc_{OT}$; **4**, $Gal \cdot Fuc \cdot GlcNAc \cdot Man_3 \cdot GlcNAc \cdot Fuc \cdot GlcNAc_{OT}$; **5**, $Man_3 \cdot GlcNAc \cdot Fuc \cdot GlcNAc_{OT}$; **6**, $Gal_2 \cdot GlcNAc_2 \cdot Man_2 \cdot GlcNAc \cdot Man \cdot GlcNAc \cdot Fuc \cdot GlcNAc_{OT}$. (A) Fraction AN; (B) fraction in (A) after digestion with almond α-fucosidase I; (C) fraction I in (A) after digestion with a mixture of jack bean β-galactosidase and jack bean β-*N*-acetylhexosaminidase; (D) peak *d* in (C) after digestion with almond α-fucosidase I; (E) the peak in (D) after digestion with *A. saitoi* α-mannosidase II (solid line) or after sequential digestion with diplococcal β-galactosidase and diplococcal β-*N*-acetylhexosaminidase (dotted line); (F) fraction II in (A) after digestion with a mixture of jack bean β-galactosidase and jack bean β-*N*-acetylhexosaminidase; (G) peak *f* in (F) after sequential digestion with almond α-fucosidase I (solid line), diplococcal β-galactosidase (dotted line), and *A. saitoi* α-mannosidase II (dot-dash line); (H) peak *g* in (F) after sequential digestion with almond α-fucosidase I (solid line) and diplococcal β-galactosidase (dotted line).

These results indicated that the sialic acid residues of the three acidic fractions (A1–3) are linked both at the C-6 and at the C-3 positions of the terminal galactose residues.

It is noteworthy that neither 3,6- nor 3,4-di-*O*-methylmannitol was detected in fraction N. This result indicated that fraction N does not contain any tri- and tetraantennary oligosaccharides.

Structural Studies of Oligosaccharides in Fraction AN by Exoglycosidase Digestion. To determine the anomeric configuration and the monosaccharide sequence in each oligosaccharide, radioactive oligosaccharides in fraction AN were subjected to sequential exoglycosidase digestion, and the reaction product at each step was analyzed by Bio-Gel P-4 column chromatography. Although the elution profile of fraction AN on Bio-Gel P-4 column chromatography was quite heterogeneous (Figure 3A), it was separated into three peaks after almond α-fucosidase I digestion (peaks *a*–*c* in Figure 3B) in a molar ratio of 11:67:22.

Table I: Methylation Analysis of Fractions N, A, and AN Obtained from PrP 27–30

methylated sugar	molar ratio[a] N	A	AN
fucitol			
2,3,4-tri-*O*-methyl (1,5-di-*O*-acetyl)	1.5	1.6	1.7
galactitol			
2,3,4,6-tetra-*O*-methyl (1,5-di-*O*-acetyl)	0.7	1.2	2.8
2,4,6-tri-*O*-methyl (1,3,5-tri-*O*-acetyl)	*b*	0.5	*b*
2,3,4-tri-*O*-methyl (1,5,6-tri-*O*-acetyl)	*b*	1.1	*b*
mannitol			
3,4,6-tri-*O*-methyl (1,2,5-tri-*O*-acetyl)	2.0	1.0	1.1
3,6-di-*O*-methyl (1,2,4,5-tetra-*O*-acetyl)	*b*	0.7	0.7
3,4-di-*O*-methyl (1,2,5,6-tetra-*O*-acetyl)	*b*	0.1	0.1
2-mono-*O*-methyl (1,3,4,5,6-penta-*O*-acetyl)	1.0	1.0	1.0
2-(*N*-methylacetamido)-2-deoxyglucitol			
1,3,5-tri-*O*-methyl (4,6-di-*O*-acetyl)	0.9	0.8	0.9
3,4,6-tri-*O*-methyl (1,5-di-*O*-acetyl)	2.2	0.9	0.9
3,6-di-*O*-methyl (1,4,5-tri-*O*-acetyl)	1.0	2.8	2.9
6-mono-*O*-methyl (1,3,4,5-tetra-*O*-acetyl)	0.6	0.8	0.9

[a] Numbers in the table were calculated by taking the value of 2-mono-*O*-methylmannitol as 1.0. [b] Not detected.

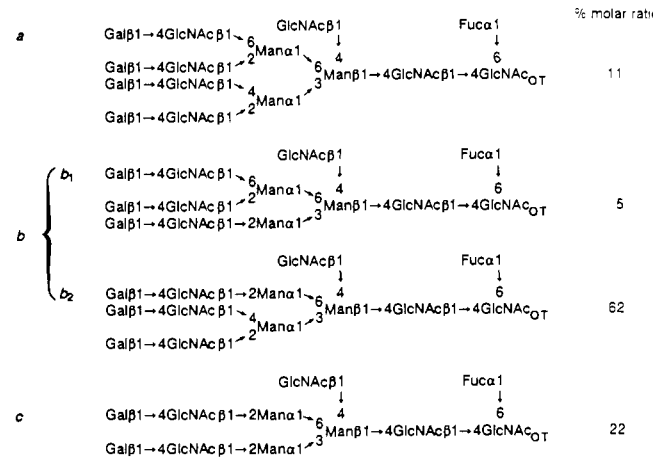


FIGURE 4: Structures of defucosylated oligosaccharides from fraction AN and their percentage molar ratio.

Structures of oligosaccharides in peaks *a* and *c* were elucidated as shown in Figure 4. Since the analytical procedures to reach these structural assignments were the same as reported in the previous papers (Yamashita et al., 1983, 1985, 1986), they will not be described here. That oligosaccharide b_1 and/or oligosaccharide b_2 in Figure 4 was included in peak *b* was confirmed by sequential exoglycosidase digestion as reported previously (Renwick et al., 1987; Yamashita et al., 1983, 1985, 1986) (data not shown). When peak *b* was applied to a DSA–Sepharose column, it was separated into two fractions, a retarded and a bound fraction in the molar ratio 93:7 (data

not shown). On the basis of the binding specificity of the DSA–Sepharose column (Yamashita et al., 1987), it was concluded that peak *b* contains oligosaccharides b_1 and b_2 in Figure 4 in the molar ratio 7:93. The percentage molar ratio of the four identified oligosaccharides is also given in Figure 4.

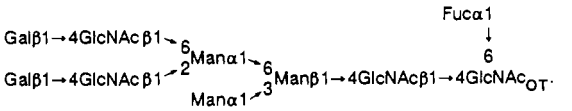
It is well-known that almond α-fucosidase I cleaves the α-fucosyl linkages of the Galβ1→4(Fucα1→3)GlcNAc and the Galβ1→3(Fucα1→4)GlcNAc groups and not of the Fucα1→2Gal group (Ogata et al., 1977). As described above, the oligosaccharide mixtures in defucosylated fraction AN contain only the Galβ1→4GlcNAc linkage. Therefore, it was concluded that fraction AN is a mixture of bi-, tri-, and tetraantennary complex-type oligosaccharides with the Galβ1→4GlcNAcβ1→ and Galβ1→4(Fucα1→3)GlcNAcβ1→ groups in their outer chain moieties.

To determine the location of the Galβ1→4(Fucα1→3)GlcNAcβ1→ group in the outer chain moieties of oligosaccharides in AN, fractions I and II (indicated by bars in Figure 3A) were separately pooled and digested with a mixture of jack bean β-galactosidase and jack bean β-*N*-acetylhexosaminidase to remove completely the nonfucosylated outer chain moieties. That fraction I contained only the biantennary complex-type oligosaccharides and fraction II contained both the tri- and tetraantennary complex-type oligosaccharides was estimated from the effective sizes of radioactive peaks in the gel permeation chromatogram (Yamashita et al., 1982). On the basis of the radioactivities, the molar ratio of fractions I and II was calculated to be 22:78. By the enzymatic treatment, fraction I was degraded into two components (Figure 3C) and fraction II into three components (Figure 3F), and the percentage molar ratio of the five components was peak *d* (13%), peak *e* (9%), peak *f* (16%), peak *g* (45%), and peak *h* (17%).

Peak *e* in Figure 3C was eluted at the same position as authentic Man_3·GlcNAc·Fuc·$GlcNAc_{OT}$. This component should be derived from the biantennary complex-type oligosaccharides without any Galβ1→4(Fucα1→3)GlcNAc outer chain because β-galactosidase cannot hydrolyze the β-galactosyl residue of this trisaccharide group. Therefore, peak *e* should be derived from oligosaccharide **1** in the summary Figure 6.

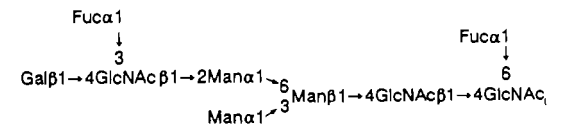
Peak *d*, which eluted at the same position as authentic Gal·Fuc·GlcNAc·Man_3·GlcNAc·Fuc·$GlcNAc_{OT}$, released a fucose residue by almond α-fucosidase I digestion (Figure 3D). The radioactive peak in Figure 3D released one galactose and one *N*-acetylglucosamine residue by sequential digestion with diplococcal β-galactosidase and diplococcal β-*N*-acetylhexosaminidase and moved to the same position as Man_3·GlcNAc·Fuc·$GlcNAc_{OT}$ (Figure 3E, dotted line). This result indicated that the structure of the radioactive peak in Figure 3D is Galβ1→4GlcNAcβ1→2Manα1→6 and/or 3-(Manα1→3 and/or 6)Manβ1→4GlcNAcβ1→4(Fucα1→6)-$GlcNAc_{OT}$. The radioactive peak in Figure 3D released one mannose residue by *A. saitoi* α-mannosidase II digestion (Figure 3E, solid line). Since this enzyme removes a mannose residue from the Galβ1→4GlcNAcβ1→2Manα1→6-(Manα1→3)Manβ1→4GlcNAc- group but not from the Manα1→6(Galβ1→4GlcNAcβ1→2Manα1→3)Manβ1→-4GlcNAc- group (Amano & Kobata, 1986), the radioactive peak in Figure 3D should contain Galβ1→4GlcNAcβ1→-2Manα1→6(Manα1→3)Manβ1→4GlcNAcβ1→4(Fucα1→-6)$GlcNAc_{OT}$ only. In peak *d*, the Galβ1→4GlcNAcβ1→ outer chain should occur as the Galβ1→4(Fucα1→3)GlcNAc group. Therefore, peak *d* should be derived from oligosaccharide **2** shown in Figure 6.

Radioactive peak *f* in Figure 3F released two fucose residues by almond α-fucosidase I digestion (Figure 3G, solid line). The radioactive solid-line product in Figure 3G released two galactosyl groups by incubation with diplococcal β-galactosidase (Figure 3G, dotted line). When the dotted-line product in Figure 3G was incubated with *A. saitoi* α-mannosidase II, one mannose residue was released (Figure 3G, dot-dash line). The dotted-line product in Figure 3G was completely resistant to diplococcal β-*N*-acetylhexosaminidase digestion but released two *N*-acetylglucosamine residues by jack bean β-*N*-acetylhexosaminidase digestion and moved to the same position as Man_3·GlcNAc·Fuc·$GlcNAc_{OT}$ (data not shown). When the solid-line component shown in Figure 3G was subjected to a DSA–Sepharose column, it was bound to the column and then eluted with the buffer containing *N*-acetylglucosamine oligomers (data not shown). These results indicated that the solid-line peak in Figure 3G contains the oligosaccharide



The two Galβ1→4GlcNAc outer chains should be fucosylated in peak *f* because these two galactose residues were resistant to β-galactosidase treatment before defucosylation. As described already, oligosaccharides containing the Galβ1→4GlcNAcβ1→6(Galβ1→4GlcNAcβ1→2)Man group accounted for 16% of fraction AN (Figure 4). Since the molar ratio of peak *f* in fraction AN was 16%, all oligosaccharides containing the 2,6-branch in fraction AN should have been converted to peak *f*. On the basis of these results, it was concluded that all 2,6-branched outer chains of tetraantennary and triantennary oligosaccharides are completely fucosylated. Therefore, oligosaccharides *a* and b_1 in Figure 4 should occur in fraction AN as oligosaccharides **6** and **5** in Figure 6, respectively.

The data described above also indicated that another two products in Figure 3F (peaks *g* and *h*) should be derived from 2,4-branched triantennary oligosaccharides. When peak *g* in Figure 3F was incubated with almond α-fucosidase I, it released a fucose residue (Figure 3H, solid line). The radioactive solid-line product in Figure 3H released one galactose residue by incubation with diplococcal β-galactosidase (Figure 3H, dotted line). The dotted-line product in Figure 3H released a mannose residue by *A. saitoi* α-mannosidase II digestion, and it also released an *N*-acetylglucosamine residue by diplococcal β-*N*-acetylhexosaminidase digestion (data not shown). These results indicated that peak *g* in Figure 3F contains the oligosaccharide



This nonasaccharide should be derived from oligosaccharide **4** in Figure 6.

Peak *h* in Figure 3F was eluted at the same position as authentic Man_3·GlcNAc·Fuc·$GlcNAc_{OT}$ and was concluded to be derived from oligosaccharide **3** in Figure 6.

The analytical data so far described indicated that oligosaccharides **3**–**6** in Figure 6 are included in fraction II in Figure 3A.

Structures of the Oligosaccharides in Fraction N. Upon Bio-Gel P-4 column chromatography, fraction N gave a complicated elution profile as shown in Figure 5A. By incubation with almond α-fucosidase I, a part of the oligo-

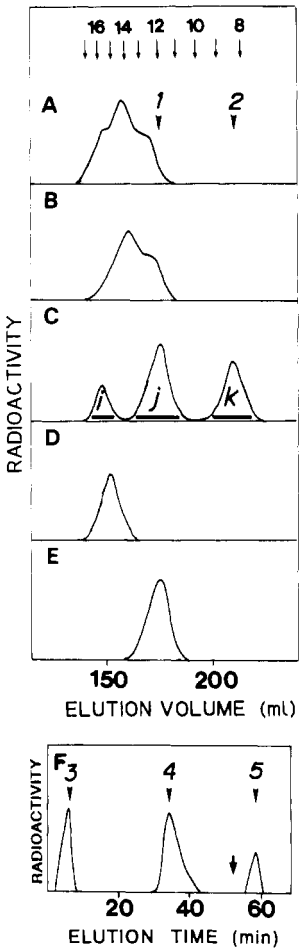


FIGURE 5: Bio-Gel P-4 column chromatography of oligosaccharide fraction N and its sequential exoglycosidase digestion products (A–E) and RCA-HPLC column chromatogram of fraction N (F). Arrows in (A) are the same as in Figure 3. Arrow heads in (A) and (F) indicate the elution positions of authentic oligosaccharides: **1**, Gal·GlcNAc·Man_3·GlcNAc·Fuc·$GlcNAc_{OT}$; **2**, Man_3·GlcNAc·Fuc·$GlcNAc_{OT}$; **3**, $GlcNAc_2$·Man_2·GlcNAc·Man·GlcNAc·Fuc·$GlcNAc_{OT}$; **4**, Gal·$GlcNAc_2$·Man_2·GlcNAc·Man·GlcNAc·Fuc·$GlcNAc_{OT}$; **5**, Gal_2·$GlcNAc_2$·Man_2·GlcNAc·Man·GlcNAc·Fuc·$GlcNAc_{OT}$. (A) Fraction N; (B) fraction N after digestion with almond α-fucosidase I; (C) fraction N after digestion with jack bean β-*N*-acetylhexosaminidase; (D) peak *i* in (C) after digestion with diplococcal β-galactosidase; (E) the peak in (D) after digestion with jack bean β-*N*-acetylhexosaminidase. Arrow in (F) indicates the position where the elution buffer was switched to the buffer containing 10 mM lactose.

saccharide seemed to be degraded (Figure 5B). When the radioactive products in Figure 5B were subjected to an RCA-HPLC column (Harada et al., 1987), three fractions (a passed-through, a retarded, and a bound) were obtained in the percent molar ratio of 39, 50, and 11 (Figure 5F). On the basis of the specificity of the RCA-HPLC column and the results of sequential exoglycosidase digestion as reported by Harada et al. (1987), the passed-through fraction and the bound fraction in Figure 5F were found to contain oligosaccharides **7** and **1** shown in Figure 6, respectively (data not shown). The retarded fraction in Figure 5F was found to contain Galβ1→4GlcNAcβ1→2Manα1→6(GlcNAcβ1→4)(GlcNAcβ1→2Manα1→3)Manβ1→4GlcNAcβ1→4(Fucα1→6)$GlcNAc_{OT}$ by the same series of experiments reported by Harada et al. (1987). Some of their outer chain moieties should originally contain an α-fucosyl residue as the Galβ1→4(Fucα1→3)GlcNAcβ1→ group.

To determine the position of the α-fucosyl residues in the outer chain moieties, the radioactive fraction shown in Figure

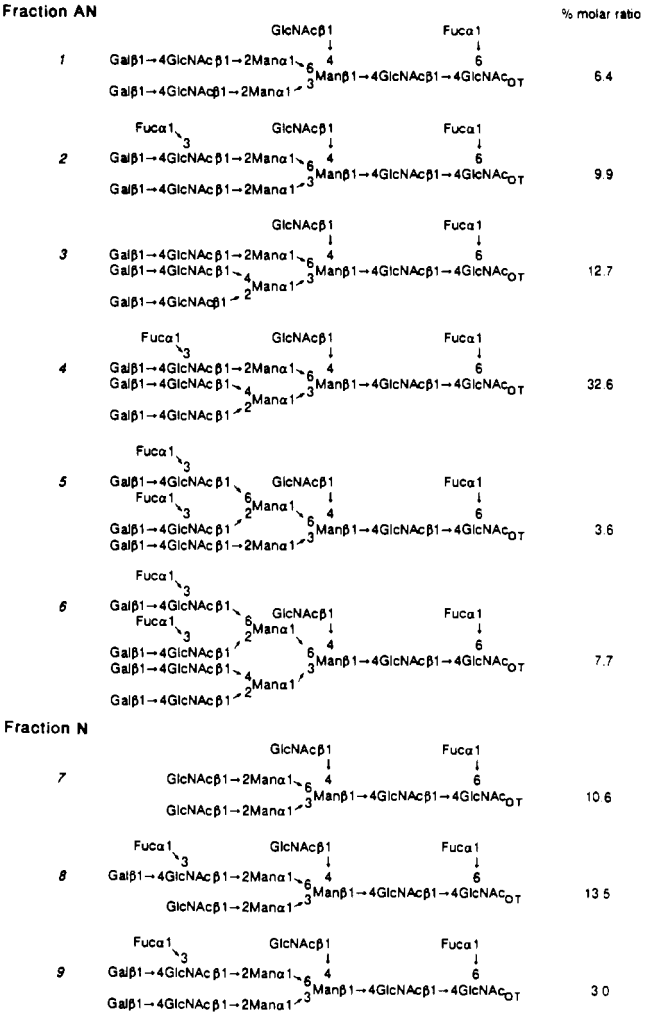


FIGURE 6: Proposed structures for the asparagine-linked oligosaccharides of scrapie prion protein, PrP 27–30, and their percentage molar ratio to total oligosaccharides.

5A was digested with jack bean β-*N*-acetylhexosaminidase. Three peaks (*i*–*k* in Figure 5C) were obtained upon Bio-Gel P-4 column chromatography, and the molar ratio of peaks *i*, *j*, and *k* was 11:50:39. The mobility of the radioactive peak *k* in Figure 5C was the same as authentic Man_3·GlcNAc·Fuc·$GlcNAc_{OT}$. This hexasaccharide should be derived from oligosaccharide **7** in Figure 6. Peak *j* with mobility of 11.9 glucose units in Figure 5C showed exactly the same results of sequential exoglycosidase digestion as those of the peak *g* in Figure 3F. Therefore, peak *j* in Figure 5C should be derived from oligosaccharide **8** in Figure 6.

Radioactive peak *i* with mobility of 15.7 glucose units in Figure 5C released one galactose and two *N*-acetylglucosamine residues by sequential digestion with diplococcal β-galactosidase (Figure 5D) and jack bean β-*N*-acetylhexosaminidase (Figure 5E), respectively. The radioactive product in Figure 5E gave the same results as peak *j* in Figure 5C by sequential exoglycosidase digestion (data not shown). Therefore, peak *i* in Figure 5C should contain oligosaccharide **9** in Figure 6. That the bisecting *N*-acetylglucosamine residue linked to digalactosylated biantennary oligosaccharide was not digested by jack bean β-*N*-acetylhexosaminidase was already reported by Taniguchi et al. (1985).

Structures of the Asparagine-Linked Oligosaccharides of PrP 27–30. Our data indicate that PrP 27–30 contains the nine oligosaccharides in the percent molar ratio shown in Figure 6. As described in the methylation analysis (Table I), some of the galactose residues in oligosaccharides **1**–**6**

should occur as the Siaα2→3Gal and Siaα2→6Gal groups. On the basis of the structures in Figure 6, approximately 70% of the galactose residues in oligosaccharides **1–6** occur as the Galβ1→4GlcNAcβ1→ group and the remainder as the Galβ1→4(Fucα1→3)GlcNAcβ1→ group. The methylation data of fraction A in Table I indicated that more than 30% of the total galactose residues in this fraction remained nonsialylated. Therefore, the fucosylated outer chain may not be sialylated as in the case of human parotid amylase (Yamashita et al., 1980) and mouse kidney γ-glutamyltranspeptidase (Yamashita et al., 1985).

Discussion

While the scrapie isoform of PrP is clearly a necessary component of the infectious prion particle (Gabizon et al., 1987; Prusiner, 1987), the function of PrP^C is unknown. Both PrP^{Sc} and PrP^C in hamsters are encoded by an ORF of 254 amino acids (Basler et al., 1987; Prusiner, 1986). During biosynthesis both proteins lose a 22 amino acid signal peptide from their N-termini (Basler et al., 1986; Robakis et al., 1986; Hope et al., 1988; Turk et al., 1988). In addition, a highly hydrophobic C-terminal peptide appears to be removed when a glycosylphosphatidylinositol (GPI) anchor is added to both isoforms (Stahl et al., 1987). Codons 181–183 have the sequence Asn-Ile-Thr (NIT) and codons 197–199 Asn-Phe-Thr (NFT), both of which are consensus sequences for asparagine-linked glycosylation (Pless & Lennarz, 1977; Hart et al., 1979). The data reported here indicate that the oligosaccharide pattern of the scrapie prion protein PrP 27–30 is very complex and diverse. Indeed, calculation of the possible oligosaccharides (Figure 6) with zero (fraction N), one (fraction A1), two (fraction A2), or three (fraction A3) sialic acid residues (Figure 2) yields 401 forms of PrP^{Sc} when both sites are glycosylated. This extreme variation may be one of the reasons for the broad band observed with PrP 27–30 on SDS–PAGE (Bolton et al., 1982; Prusiner et al., 1982).

In other papers (Bolton et al., 1985; Manuelidis et al., 1985; Haraguchi et al., unpublished observations), the information about the structures of the oligosaccharides attached on prion proteins was obtained from lectin affinity chromatography and the susceptibility to sialidase and endoglycosidases (endoglycosidase H and *N*-glycanase). Only complex-type oligosaccharides were detected attached to the prion protein; these oligosaccharides were resistant to endoglycosidase H digestion but sensitive to *N*-glycanase digestion. It was concluded from the results of kinetic studies with *N*-glycanase digestion that the scrapie prion protein contains two asparagine-linked oligosaccharides. It is noteworthy that cell-free translation studies using either reticulocyte lysates or wheat germ extracts and dog pancreas microsomes with recombinant PrP mRNA produced PrP molecules that were sensitive to endoglycosidase H digestion (Hay et al., 1987). Not unexpectedly, these high-mannose oligosaccharides are modified in the Golgi in vivo to produce authentic PrP^C and PrP^{Sc} that are resistant to endoglycosidase H but susceptible to *N*-glycanase. Since sialidase digestion of PrP 27–30 reduced the number of acidic charge isomers, it was thought to be a sialoglycoprotein (Bolton et al., 1985). These sialic acid residues were thought to be linked to galactose residues because PrP 27–30 did not bind to an RCA–Sepharose column but bound to the column after sialidase treatment (Haraguchi et al., unpublished observations). The oligosaccharides of PrP 27–30 were also thought to contain a fucosylated trimannosyl core because PrP 27–30 bound to a lentil lectin–Sepharose column.

The foregoing features of PrP 27–30 oligosaccharides are supported by the structural studies reported here. Two moles of complex-type asparagine-linked oligosaccharides was found linked to the polypeptide portion of PrP 27–30. It will be of interest to learn if PrP molecules from other species also contain 2 mol of complex-type asparagine-linked oligosaccharides. Molecular cloning and sequencing of PrP genes from human, Syrian hamster, Chinese hamster, Armenian hamster, three strains of mice, rat, and sheep indicate that all these species have PrP genes with two consensus sites for asparagine-linked glycosylation in the C-terminal domain. These highly conserved glycosylation sites argue that the PrP oligosaccharides are essential for the function of PrP^C. More than 70% of hamster PrP 27–30 oligosaccharides were sialylated, and the sialic acid residues were linked to penultimate galactose residues. The core domains of these oligosaccharides were all fucosylated.

Besides quantitating the level of glycosylation and validating several structural features, many new findings include our discovery that all PrP 27–30 oligosaccharides contain bisecting *N*-acetylglucosamine residues, and a portion of the oligosaccharides contain the Galβ1→4(Fucα1→3)GlcNAc group (X-antigenic determinant) in their outer chain moieties.

X-Antigenic determinant is widely distributed in various tissues and cell types as free oligosaccharides (Kobata & Ginsburg, 1969; Hallgren & Lundblad, 1977), glycolipids (Yang & Hakomori, 1971), and glycoproteins (Yamashita et al., 1980, 1985; Endo et al., 1986). This trisaccharide is also designated as stage-specific embryonic antigen 1 (SSEA-1), and its expression on cell surface in early developmental stage is considered to function as adhesion molecules between the cell–cell interactions in normal developmental processes in embryo (Solter & Knowles, 1978; Fenderson et al., 1984). Finne et al. (1980) reported that it is expressed only on poorly metastasizing wheat germ agglutinin (WGA) resistant melanoma cells and not on highly metastasizing parental B16 mouse melanoma cells. These results suggested that the altered glycosylation, including the appearance or disappearance of the X-antigenic determinant on the cell surface, may play an important role in the cell to cell recognition phenomena.

It is of interest that PrP^C synthesis is developmentally regulated in the brains of newborn hamsters (McKinley et al., 1987). PrP gene transcription increases at different rates in various regions of the Syrian hamster brain during the first 2 weeks after birth (Mobley et al., 1988). In addition, PrP^C is found almost exclusively on the external surface of cells where it is bound by a GPI anchor (Stahl et al., 1987). These observations taken together with the X-antigenic determinant of PrP^{Sc} asparagine-linked oligosaccharides suggest that PrP^C may function as a cell recognition or adhesion molecule. Whether PrP^C contains the same asparagine-linked oligosaccharides as PrP^{Sc} remains to be established. Enzymatic and chemical deglycosylation studies of PrP^C suggest that its asparagine-linked oligosaccharides have also complex-type structures as those reported here for PrP^{Sc} (Haraguchi et al., unpublished observations).

Reports of the bisected complex-type sugar chains with the X-antigenic determinant in their outer chain moieties are limited. So far, these oligosaccharides were detected in human (Yamashita et al., 1986) and mouse (Yamashita et al., 1985) kidney γ-glutamyltranspeptidases and in the membrane glycoproteins of the human myeloid and monocytoid cell lineages (Mizoguchi et al., 1984).

It is of interest that the neutral fraction (N) contained only biantennary complex-type oligosaccharides and the acidic fractions (A1 plus A2 plus A3) contained not only biantennary but also tri- and tetraantennary oligosaccharides. In view of

the specific distribution of different oligosaccharides at different asparagine loci of glycoprotein molecules as clearly demonstrated in human chorionic gonadotropin (hCG) (Mizuochi & Kobata, 1980) and bovine blood coagulation factors II and IX (Berger et al., 1982; Mizuochi et al., 1983), it is possible that the bi-, tri-, and tetraantennary oligosaccharides are distributed at different asparagine loci of PrP 27–30.

Whether differences in asparagine-linked oligosaccharide structure account for the different properties exhibited by PrP^{C} and PrP^{Sc} remains to be established. Equally interesting is the possibility that the diversity of PrP^{Sc} oligosaccharide structures features in the different biological properties exhibited by distinct "strains" (Dickinson et al., 1979) or isolates of scrapie prions (Prusiner, 1987). That small structural changes in the prion protein can drastically alter scrapie incubation times in mice comes from recent studies where PrP^{Sc}-A prions were found to yield incubation time of ~280 days in mice, while PrP^{Sc}-B prions gave ~190 days. PrP^{Sc}-A differs from PrP^{Sc}-B by two amino acids at codons 108 and 189 (Westaway et al., 1987; Carlson et al., unpublished observations).

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References

Amano, J., & Kobata, A. (1986) *J. Biochem. (Tokyo) 99*, 1645–1654.

Basler, K., Oesch, B., Scott, M., Westaway, D., Wälchli, M., Groth, D. F., McKinley, M. P., Prusiner, S. B., & Weissmann, C. (1986) *Cell 46*, 417–428.

Berger, E. G., Buddecke, E., Kamerling, J. P., Kobata, A., Paulson, J. C., & Vligenthart, J. F. G. (1982) *Experientia 38*, 1129–1162.

Bolton, D. C., McKinley, M. P., & Prusiner, S. B. (1982) *Science 218*, 1309–1311.

Bolton, D. C., Meyer, R. K., & Prusiner, S. B. (1985) *J. Virol. 53*, 596–606.

Chesebro, B., Race, R., Wehrly, K., Nishio, J., Bloom, M., Lechner, D., Bergstrom, S., Robbins, K., Mayer, L., Keith, J. M., Garon, C., & Haase, A. (1985) *Nature 315*, 331–333.

Dickinson, A. G., & Outram, G. W. (1979) in *Slow Transmissible Disease of the Nervous System* (Prusiner, S. B., & Hadlow, W. J., Eds.) Vol. 2, pp 13–31, Academic Press, New York.

Endo, T., Amano, J., Berger, E. G., & Kobata, A. (1986) *Carbohydr. Res. 150*, 241–263.

Endo, T., Nishimura, R., Kawano, T., Mochizuki, M., & Kobata, A. (1987) *Cancer Res. 47*, 5242–5245.

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

Fenderson, B. A., Zehavi, U., & Hakomori, S. (1984) *J. Exp. Med. 160*, 1591–1596.

Finne, J., Tao, T.-W., & Burger, M. M. (1980) *Cancer Res. 40*, 2580–2587.

Gabizon, R., McKinley, M. P., & Prusiner, S. B. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 4017–4021.

Gajdusek, D. C. (1977) *Science 197*, 943–960.

Glasgow, L. R., Paulson, J. C., & Hill, R. L. (1977) *J. Biol. Chem. 252*, 8615–8623.

Hallgren, P., & Lundblad, A. (1977) *J. Biol. Chem. 252*, 1014–1022.

Harada, H., Kamei, M., Tokumoto, Y., Yui, S., Koyama, F., Kochibe, N., Endo, T., & Kobata, A. (1987) *Anal. Biochem. 164*, 374–381.

Hart, G. W., Brew, K., Grant, G. A., Bradshaw, R. A., & Lennarz, W. J. (1979) *J. Biol. Chem. 254*, 9747–9753.

Hay, B., Barry, R. A., Lieberburg, I., Prusiner, S. B., & Lingappa, V. R. (1987) *Mol. Cell. Biol. 7*, 914–920.

Hope, J., Multhaup, G., Reekie, L. J. D., Kimberlin, R. H., & Beyreuther, K. (1988) *Eur. J. Biochem. 172*, 271–277.

Jones, B. N. (1986) in *Methods of Protein Microcharacterization* (Shively, J. E., Ed.) pp 121–151, Humana Press, Clifton, NJ.

Kobata, A. (1982) *Methods Enzymol. 83*, 625–631.

Kobata, A., & Ginsburg, V. (1969) *J. Biol. Chem. 244*, 5496–5502.

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

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

Manuelidis, L., Valley, S., & Manuelidis, E. E. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 4263–4267.

Masters, C. L., Gajdusek, D. C., & Gibbs, C. J., Jr. (1981) *Brain 104*, 535–558.

McKinley, M. P., Hay, B., Lingappe, V. R., Lieberburg, I., & Prusiner, S. B. (1987) *Dev. Biol. 121*, 105–110.

Merril, C. R., Goldman, D., Sedman, S. A., & Ebert, M. H. (1981) *Science 211*, 1437–1438.

Meyer, R. K., McKinley, M. P., Bowman, K. A., Barry, R. A., & Prusiner, S. B. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 2310–2314.

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

Mizuochi, T., & Kobata, A. (1980) *Biochem. Biophys. Res. Commun. 97*, 772–778.

Mizuochi, T., Taniguchi, T., Shimizu, A., & Kobata, A. (1982) *J. Immunol. 129*, 2016–2020.

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

Mobley, W. C., Neve, R. L., Prusiner, S. B., & McKinley, M. P. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 9811–9815.

Oesch, B., Westaway, D., Wälchli, M., McKinely, M. P., Kent, S. B. H., Aebersold, R., Barry, R. A., Tempst, P., Teplow, D. B., Hood, L. E., & Prusiner, S. B. (1985) *Cell 40*, 735–746.

Ogata, M.-A., Muramatsu, T., & Kobata, A. (1977) *Arch. Biochem. Biophys. 181*, 353–358.

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

Pless, D. D., & Lennarz, W. J. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 134–138.

Prusiner, S. B. (1982) *Science 216*, 136–144.

Prusiner, S. B. (1987) *N. Engl. J. Med. 317*, 1571–1581.

Prusiner, S. B., Bolton, D. C., Groth, D. F., Bowman, K. A., Cochran, S. P., & McKinley, M. P. (1982) *Biochemistry 21*, 6942–6950.

Prusiner, S. B., McKinely, M. P., Bowman, K. A., Bolton, D. C., Bendheim, P. E., Groth, D. F., & Glenner, G. G. (1983) *Cell 35*, 349–358.

Renwick, A. G. C., Mizuochi, T., Kochibe, N., & Kobata, A. (1987) *J. Biochem. (Tokyo) 101*, 1209–1221.

Robakis, N. K., Sawh, P. R., Wolfe, C. C., Rubenstein, R., Carp, R. I., & Innis, M. A. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 6377–6381.

Smith, P. K., Krohn, R. I., Hermanson, G. T., Mallia, A. K., Gartner, F. H., Provenzano, M. D., Fujimoto, E. K., Goeke, N. M., Olson, B. J., & Klenk, D. C. (1985) *Anal. Biochem. 150*, 76–85.

Solter, D., & Knowles, B. B. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 5565-5569.
Stahl, N., Borchelt, D. R., Hsiao, K., & Prusiner, S. B. (1987) *Cell 51*, 229-240.
Takasaki, S., Mizuochi, T., & Kobata, A. (1982) *Methods Enzymol. 83*, 263-268.
Taniguchi, T., Mizuochi, T., Beale, M., Dwek, R. A., Rademacher, T. W., & Kobata, A. (1985) *Biochemistry 24*, 5551-5557.
Turk, E., Teplow, D. B., Hood, L. E., & Prusiner, S. B. (1988) *Eur. J. Biochem. 176*, 21-30.
Westaway, D., Goodman, P. A., Mirenda, C. A., McKinley, M. P., Carlson, G. A., & Prusiner, S. B. (1987) *Cell 51*, 651-662.
Yamashita, K., Tachibana, Y., Nakayama, T., Kitamura, M., Endo, Y., & Kobata, A. (1980) *J. Biol. Chem. 255*, 5635-5642.
Yamashita, K., Liang, C.-J., Funakoshi, S., & Kobata, A. (1981) *J. Biol. Chem. 256*, 1283-1289.
Yamashita, K., Mizuochi, T., & Kobata, A. (1982) *Methods Enzymol. 83*, 105-126.
Yamashita, K., Tachibana, Y., Shichi, H., & Kobata, A. (1983) *J. Biochem. (Tokyo) 93*, 135-147.
Yamashita, K., Hitoi, A., Tateishi, N., Higashi, T., Sakamoto, Y., & Kobata, A. (1985) *Arch. Biochem. Biophys. 240*, 573-582.
Yamashita, K., Hitoi, A., Matsuda, Y., Miura, T., Katunuma, N., & Kobata, A. (1986) *J. Biochem. (Tokyo) 99*, 55-62.
Yamashita, K., Totani, K., Ohkura, T., Takasaki, S., Goldstein, I. J., & Kobata, A. (1987) *J. Biol. Chem. 262*, 1602-1607.
Yang, H.-J., & Hakomori, S. (1971) *J. Biol. Chem. 246*, 1192-1200.

Hemagglutinins from Two Influenza Virus Variants Bind to Sialic Acid Derivatives with Millimolar Dissociation Constants: A 500-MHz Proton Nuclear Magnetic Resonance Study†

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ABSTRACT: The equilibrium binding of influenza virus hemagglutinin to derivatives of its cell-surface ligand, sialic acid, was measured by nuclear magnetic resonance (NMR) spectroscopy. Binding was quantified by observing perturbations of sialic acid resonances in the presence of protein. The major perturbation observed was a chemical shift of the *N*-acetyl methyl resonance, presumably due to the proximity of the methyl group to tryptophan 153. X-31 hemagglutinin binds to the methyl α-glycoside of sialic acid with a dissociation constant of 2.8 mM and does not bind to the methyl β-glycoside. Replacing the 4-hydroxyl group of sialic acid with an acetyl group has little effect, while replacing the 7-hydroxyl group with an acetyl prevents binding. Experiments with sialylated oligosaccharides confirm literature reports that mutations at amino acid 226 change the specificity of hemagglutinin for $\alpha(2,6)$ and $\alpha(2,3)$ glycosidic linkages. The NMR line broadening of sialyloligosaccharides suggests that sialic acid is the only component that contacts the protein. Saccharides containing two sialic acid residues appear to have two separate binding modes. Hemagglutinin that has undergone a low pH induced conformational change retains the ability to bind sialic acid.

Influenza virus infection is initiated by the attachment of virus particles to cell-surface receptors containing sialic acid [reviewed in Wiley and Skehel (1987)]. Sialic acid is recognized by the viral glycoprotein hemagglutinin, a membrane-bound trimer consisting of three HA1 and three HA2 polypeptide chains. Viral attachment is followed by receptor-mediated endocytosis, after which the viral and cell membranes fuse, allowing the viral nucleocapsid to enter the cytoplasm. Membrane fusion is thought to be mediated by a conformational change in the hemagglutinin, triggered at the pH of the endosome.

The recently published structure of hemagglutinin complexed with sialyllactose at 3-Å resolution (Weis et al., 1988) reveals that sialic acid binds to a shallow pocket in the HA1 polypeptide, a pocket containing several conserved amino acid residues. Amino acid mutations in or near the binding pocket are known to change the hemagglutinin's binding properties. Viruses that contain leucine at position 226, for example, preferentially agglutinate erythrocytes that possess sialic acids joined in an $\alpha(2,6)$ linkage with galactose, whereas viruses

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