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THE STRUCTURE OF THE ASPARAGINE-LINKED SUGAR CHAINS OF BOVINE BRAIN RIBONUCLEASE

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ABSTRACT

The asparagine-linked sugar chains of bovine brain ribonuclease were quantitatively released as oligosaccharides from the polypeptide backbone by hydrazinolysis. After *N*-acetylation, they were converted into radioactively-labeled oligosaccharides by NaB^3H_4 reduction. The radioactive oligosaccharide mixture was fractionated by ion-exchange chromatography, and the acidic oligosaccharides were converted into neutral oligosaccharides by sialidase digestion. The neutral oligosaccharides were then fractionated by Bio-Gel P-4 column chromatography. Structural studies of each oligosaccharide by sequential exoglycosidase digestion in combination with methylation analysis revealed that bovine brain ribonuclease showed extensive heterogeneity. It contains bi- and tri-antennary, complex-type oligosaccharides having α-D-Man*p*-(1→3)-[α-D-Man*p*-(1→6)]-β-D-Man*p*-(1→4)-β-D-Glc*p*NAc-(1→4)-[α-L-Fuc*p*-(1→6)]-D-GlcNAc as their common core. Four different outside oligosaccharide chains, *i.e.*, β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc-(1→, α-Neu5Ac-(2→6)-β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc-(1→, α-Neu5Ac-(2→3)-β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc-(1→, and α-D-Gal*p*-(1→3)-β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc-(1→, were found. The preferential distribution of the α-D-Gal*p*-(1→3)-β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc group on the α-D-Man*p*-(1→6) arm is a characteristic feature of the sugar chains of this enzyme.

INTRODUCTION

Ribonucleases (RNases), many of which have been shown to be glycoproteins, are widely distributed in various organs of mammals[1]. Four RNases, A, B, C, and D, which can be separated by ion-exchange column chromatography, were found to occur in bovine pancreas[2,3]. Because the polypeptide portions of these enzymes are the same, their heterogeneity should be ascribed to the difference in their

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carbohydrate components[2–4]. Elson and Gliz[5] have purified RNase from bovine brain and analyzed its enzymic properties. It is a glycoprotein containing 9% of carbohydrate. As a part of the studies to elucidate the complete structure of this enzyme, we have investigated its sugar component.

EXPERIMENTAL

Chemicals and enzymes. — All chemicals used in this study were of reagent grade. NaB^3H_4 (1.18 TBq/mol) was purchased from New England Nuclear, Boston, MA. NaB^2H_4 (98%) was obtained from Merck Co., Darmstadt, FRG. β-D-Galactosidase (EC 3.2.1.23), *N*-acetyl-β-D-hexosaminidase (EC 3.2.1.52), and α-D-mannosidase (EC 3.2.1.24) were purified from jack bean meal according to the method of Li and Li[6]. β-D-Galactosidase and *N*-acetyl-β-D-hexosaminidase were also purified from the culture fluid of *Diplococcus pneumoniae* according to the method of Glasgow *et al.*[7]. Newcastle disease virus (NDV) sialidase (EC 3.2.1.18) was purified from NDV B1 strain according to the method of Paulson *et al.*[8]. Snail β-D-mannosidase (EC 3.2.1.25) was purchased from Seikagaku Kogyo Co., Tokyo. *Arthrobacter ureafaciens* sialidase was purchased from Nakarai Chemicals, Ltd., Kyoto. Bovine epididymal α-L-fucosidase (EC 3.2.1.51) was purchased from Sigma Chemical Co., St. Louis, MO. Green coffee bean α-D-galactosidase (EC 3.2.1.22) was purchased from Boehringer–Mannheim Yamanouchi Co., Tokyo. *Aspergillus saitoi* α-D-mannosidase II was purified by the method of Amano and Kobata[9]. Concanavalin A (Con A)-Sepharose[10] and *Datura stramonium* agglutinin (DSA)-Sepharose[11] columns were prepared according to the cited references.

*Oligosaccharides**. — α-Sia-(2→6)-β-Gal-(1→4)-β-GlcNAc-(1→2)-α-Man-(1→6)-[α-Sia-(2→6)-β-Gal-(1→4)-β-GlcNAc-(1→2)-α-Man-(1→3)]-β-Man-(1→4)-β-GlcNAc-(1→4)-[α-Fuc-(1→6)]-$GlcNAc_{OT}$ (Sia_2→Gal_2→$GlcNAc_2$→Man_3→GlcNAc→Fuc→$GlcNAc_{OT}$) and α-Sia-(2→6)-β-Gal-(1→4)-β-GlcNAc-(1→2)-α-Man-(1→6)-[β-Gal-(1→4)-β-GlcNAc-(1→2)-α-Man-(1→3)]-β-Man-(1→4)-β-GlcNAc-(1→4)-[α-Fuc-(1→6)]-$GlcNAc_{OT}$ (Sia→Gal_2→$GlcNAc_2$→Man_3→GlcNAc→Fuc→$GlcNAc_{OT}$) were prepared from Bence Jones protein Sm λ (ref. 12). α-Gal-(1→3)-β-Gal-(1→4)-β-GlcNAc-(1→2)-α-Man-(1→6)-[α-Gal-(1→3)-β-Gal-(1→4)-β-GlcNAc-(1→2)-α-Man-(1→3)]-β-Man-(1→4)-β-GlcNAc-(1→4)-[α-Fuc-(1→6)]-$GlcNAc_{OT}$ (Gal_2→Gal_2→$GlcNAc_2$→Man_3→GlcNAc→Fuc→$GlcNAc_{OT}$) and α-Gal-(1→3)-β-Gal-(1→4)-β-GlcNAc-(1→2)-α-Man-(1→6)-[β-Gal-(1→4)-β-GlcNAc-(1→2)-α-Man-(1→3)]-β-Man-(1→4)-β-GlcNAc-(1→4)-[α-Fuc-(1→6)]-$GlcNAc_{OT}$ (Gal→Gal_2→$GlcNAc_2$→Man_3→GlcNAc→Fuc→$GlcNAc_{OT}$) were prepared from bovine complement Clq (refs. 13, 14). β-Gal-(1→4)-β-GlcNAc-(1→2)-α-Man-(1→6)-[β-Gal-(1→4)-β-GlcNAc-(1→2)-α-Man-(1→3)]-β-Man-(1→4)-β-GlcNAc-(1→4)-[α-Fuc-(1→6)]-$GlcNAc_{OT}$ (Gal_2→$GlcNAc_2$→Man_3→GlcNAc→Fuc→Glc-

*All sugars in this paper have the D configuration, except for fucose which has the L configuration, and the pyranose ring form; the subscript OT is used to indicate NaB^3H_4-reduced oligosaccharides.

NAc_{OT}), β-GlcNAc-(1→2)-α-Man-(1→6)-[β-GlcNAc-(1→2)-α-Man-(1→3)]-β-Man-(1→4)-β-GlcNAc-(1→4)-[α-Fuc-(1→6)]-$GlcNAc_{OT}$ ($GlcNAc_2$→Man_3→GlcNAc→Fuc→$GlcNAc_{OT}$-I) and α-Man-(1→6)-[α-Man-(1→3)]-β-Man-(1→4)-β-GlcNAc-(1→4)-[α-Fuc-(1→6)]-$GlcNAc_{OT}$ (Man_3→GlcNAc→Fuc→$GlcNAc_{OT}$) were obtained from Gal_2→Gal_2→$GlcNAc_2$→Man_3→GlcNAc→Fuc→$GlcNAc_{OT}$ by sequential digestion with coffee bean α-D-galactosidase, jack bean β-D-galactosidase, and jack bean *N*-acetyl-β-D-hexosaminidase, respectively. β-Gal-(1→4)-β-GlcNAc-(1→2)-α-Man-(1→6)-{β-Gal-(1→4)-β-GlcNAc-(1→4)-[β-Gal-(1→4)-β-GlcNAc-(1→2)]-α-Man-(1→3)}-β-Man-(1→4)-β-GlcNAc-(1→4)-[α-Fuc-(1→6)]-$GlcNAc_{OT}$ (Gal_3→$GlcNAc_3$→Man_3→GlcNAc→Fuc→$GlcNAc_{OT}$-I) and β-Gal-(1→4)-β-GlcNAc-(1→6)-[β-Gal-(1→4)-β-GlcNAc-(1→2)]-α-Man-(1→6)-[β-Gal-(1→4)-β-GlcNAc-(1→2)-α-Man-(1→3)]-β-Man-(1→4)-β-GlcNAc-(1→4)-[α-Fuc-(1→6)]-$GlcNAc_{OT}$ (Gal_3→$GlcNAc_3$→Man_3→GlcNAc→Fuc→$GlcNAc_{OT}$-II) were prepared from human urinary RNase U_L (ref. 15). β-GlcNAc-(1→2)-α-Man-(1→6)-{β-GlcNAc-(1→4)-[β-GlcNAc-(1→2)]-α-Man-(1→3)}-β-Man-(1→4)-β-GlcNAc-(1→4)-[α-Fuc-(1→6)]-$GlcNAc_{OT}$ ($GlcNAc_3$→Man_3→GlcNAc→Fuc→$GlcNAc_{OT}$) and α-Man-(1→6)-[β-GlcNAc-(1→4)-α-Man-(1→3)]-β-Man-(1→4)-β-GlcNAc-(1→4)-[α-Fuc-(1→6)]-$GlcNAc_{OT}$ (GlcNAc→Man_3→GlcNAc→Fuc→$GlcNAc_{OT}$) were obtained from Gal_3→$GlcNAc_3$→Man_3→GlcNAc→Fuc→$GlcNAc_{OT}$-I by sequential digestion with diplococcal β-D-galactosidase and diplococcal *N*-acetyl-β-D-hexosaminidase, respectively; β-GlcNAc-(1→6)-[β-GlcNAc-(1→2)]-α-Man-(1→6)-[α-Man-(1→3)]-β-Man-(1→4)-β-GlcNAc-(1→4)-[α-Fuc-(1→6)]-$GlcNAc_{OT}$ ($GlcNAc_2$→Man_3→GlcNAc→Fuc→$GlcNAc_{OT}$-II) was obtained from Gal_3→$GlcNAc_3$→Man_3→GlcNAc→Fuc→$GlcNAc_{OT}$-II by digestion with a mixture of diplococcal β-D-galactosidase and diplococcal *N*-acetyl-β-D-hexosaminidase. β-Gal-(1→4)-β-GlcNAc-(1→2)-α-Man-(1→3)-[α-Man-(1→6)]-β-Man-(1→4)-β-GlcNAc-(1→4)-[α-Fuc-(1→6)]-$GlcNAc_{OT}$ (Gal→GlcNAc→Man_3→GlcNAc→Fuc→$GlcNAc_{OT}$) was isolated from human chorionic gonadotropin purified from urine of patients suffering from choriocarcinoma[16].

Analytical Methods. — Bio-Gel P-4 column chromatography was performed as described by Yamashita *et al.*[17]. Anion-exchange chromatography was carried out on a fast-protein-liquid chromatography (FPLC) apparatus (Pharmacia, Uppsala, Sweden) equipped with a Mono Q HR5/5 column. The elution was performed with 5mM sodium acetate, pH 4.0, for 10 min, then with a linear gradient from 5 to 300mM sodium acetate (pH 4.0) during 30 min, and finally with a linear gradient from 300 to 500mM sodium acetate (pH 4.0) during 10 min. Chromatography was performed at room temperature at a flow rate of 1.0 mL min^{-1}. All other analytical methods, including radioactivity detection and methylation analysis, were performed as decribed previously[15].

Glycosidase digestion. — Radioactively-labeled oligosaccharides were incubated with one of the following mixtures for 18 h at 37°, unless otherwise noted: (*a*) *A. ureafaciens* sialidase digestion, 50 milliunits of enzyme in 0.1M sodium acetate buffer, pH 5.0 (50 μL); (*b*) NDV sialidase digestion, 450 milliunits of enzyme in

10mM sodium cacodylate buffer, pH 6.5 (40 μL) for 2 h; (*c*) jack bean β-D-galactosidase digestion, 0.5 unit of enzyme in 0.1M citrate phosphate buffer, pH 3.5 (50 μL); (*d*) jack bean *N*-acetyl-β-D-hexosaminidase digestion, 0.5 unit of enzyme in 0.1M citrate phosphate buffer, pH 5.0 (50 μL); (*e*) digestion with a mixture of jack bean β-D-galactosidase and *N*-acetyl-β-D-hexosaminidase, 0.5 unit of β-D-galactosidase and 1 unit of *N*-acetyl-β-D-hexosaminidase in 0.1M citrate phosphate buffer, pH 4.0 (40 μL); (*f*) jack bean α-D-mannosidase digestion, 0.5 unit of enzyme in 0.1M sodium acetate buffer, pH 4.0 (50 μL); (*g*) *A. saitoi* α-D-mannosidase II digestion, 300 microunits of enzyme in 0.1M sodium acetate buffer, pH 5.0, containing mM $CaCl_2$ (50 μL); (*h*) snail β-D-mannosidase digestion, 10 milliunits of enzyme in 50mM sodium citrate buffer, pH 4.0 (50 μL); (*i*) α-L-fucosidase digestion, 10 milliunits of enzyme in 50mM sodium citrate buffer, pH 6.0 (40 μL); (*j*) diplococcal β-D-galactosidase digestion, 4 milliunits of enzyme in 0.1M citrate phosphate buffer, pH 6.0 (50 μL); (*k*) diplococcal *N*-acetyl-β-D-hexosaminidase digestion, 10 milliunits of enzyme in 0.1M citrate phosphate buffer, pH 6.0 (40 μL); and (*l*) coffee bean α-galactosidase digestion, 0.25 units of enzyme in 0.1M citrate phosphate buffer, pH 6.5 (40 μL), containing 50 μg of D-galactono-1,4-lactone at 25°. A small amount of toluene was added to all reaction mixtures to inhibit bacterial growth during incubation. Reactions were terminated by heating the reaction mixture for 2 min in a boiling-water bath.

Liberation of the asparagine-linked sugar chain from bovine brain RNase. — RNase from bovine brain was purified by a series of column chromatographies on phospho-cellulose, APUP-agarose [5′-(4′-aminophenylphosphoryl)uridine2′(3′)-phosphate-agarose], and Sephadex G-75, as described in the preceding paper[5]. Thoroughly dried RNase (15 mg) was subjected to hydrazinolysis for 9 h as previously described[18]. An aliquot (1/5) of the oligosaccharide fraction was reduced with NaB^3H_4 and the resulting radioactive oligosaccharide fraction was purified as described previously[18].

In order to obtain the sample for methylation analysis, the remaining oligosaccharide fraction was reduced with NaB^2H_4 in the same manner as the NaB^3H_4 reduction. To facilitate detection of the oligosaccharides, one-tenth of the tritium-labeled oligosaccharide fraction was added.

In order to determine the number of asparagine-linked sugar chains in one molecule, 25 nmol of lactose was added, as an internal standard, to the oligosaccharide fraction just before the NaB^3H_4 reduction. The radioactive oligosaccharide mixture was then subjected to paper chromatography in 4:1:1 (v/v) butanol–ethanol–water. Based on the radioactivities incorporated into lactitol and the oligosaccharide mixture, and the molecular weight of RNase (23 000), it was concluded that the enzyme contains one asparagine-linked sugar chain per molecule.

Preparation of oligosaccharide fraction free from α-D-galactose residues. — In order to determine the linkage of α-D-galactosyl group, one-fourth of the total oligosaccharides for methylation analysis was digested with *A. ureafaciens* sialidase and then with coffee bean α-D-galactosidase. Both sialic acid and galactose released

were removed from the oligosaccharide fraction by paper chromatography in 5:5:1:3 (v/v) pyridine–ethyl acetate–acetic acid–water. The oligosaccharide mixture was recovered from the paper by elution with water and then subjected to methylation analysis.

RESULTS

Fractionation of oligosaccharides by anion-exchange chromatography. — The radioactively-labeled oligosaccharide mixture, obtained from bovine brain RNase by hydrazinolysis, was separated into a neutral (N) and two acidic (A1 and A2) fractions by anion-exchange chromatography on Mono Q HR5/5 column (Fig. 1A). The molar ratio of oligosaccharides in Fractions N, A1, and A2, calculated on the basis of their radioactivities, was 3:9:13. When the acidic fractions were incubated with *A. ureafaciens* sialidase, they were completely converted into neutral oligosaccharide mixtures, indicating that only sialic acids are included as the acidic residues in these oligosaccharides (Figs. 1B and C, solid line). The neutral oligosaccharide fractions obtained from Fractions A1 and A2 were named A1N and

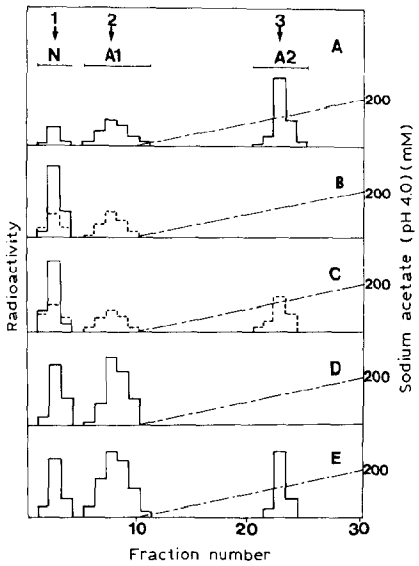


Fig. 1. Anion-exchange chromatography of the radioactive oligosaccharides. The experimental details are given in the text. Arrows indicate the positions where authentic oligosaccharides were eluted: (1) $Gal_2 \rightarrow GlcNAc_2 \rightarrow Man_3 \rightarrow GlcNAc \rightarrow Fuc \rightarrow GlcNAc_{OT}$, (2) $Sia \rightarrow Gal_2 \rightarrow GlcNAc_2 \rightarrow Man_3 \rightarrow GlcNAc \rightarrow Fuc \rightarrow GlcNAc_{OT}$, and (3) $Sia_2 \rightarrow Gal_2 \rightarrow GlcNAc_2 \rightarrow Man_3 \rightarrow GlcNAc \rightarrow Fuc \rightarrow GlcNAc_{OT}$; (A) Radioactive oligosaccharides liberated from bovine brain RNase; (B) the radioactively-labeled Fraction A1 in (A) either digested with *A. ureafaciens* sialidase (——), or partially hydrolyzed with 10mM HCl for 3 min at 100° (------); (C) the radioactive Fraction A2 in (A) either digested with *A. ureafaciens* sialidase (——), or partially hydrolyzed with 10mM HCl for 3 min at 100° (------); (D) the radioactively-labeled Fraction A1 in (A) digested with NDV sialidase; and (E) the radioactively-labeled Fraction A2 in (A) digested with NDV sialidase.

A2N, respectively. By mild acid hydrolysis (10mM HCl for 3 min at 100°), after which part of the original acidic fractions still remained, Fraction A2 gave another acidic component having the same elution position as Fraction A1 (Fig. 1C, dotted line), whereas Fraction A1 gave only a neutral component (Fig. 1B, dotted line). These results indicated that Fractions A1 and A2 contain one and two moles of sialic acid groups per molecule, respectively. Sialidase of NDV is very useful in determining the sialic acid linkages, because it cleaves the α-Sia-(2→3)-D-Gal linkage, but not the α-Sia-(2→6)-D-Gal linkage[8]. When Fraction A1 was incubated with NDV sialidase, 65.5% remained unchanged (Fig. 1D). In the case of Fraction A2, 25% remained unchanged, and 52.5% was converted into monosialyloligosaccharides (Fig. 1E). These results indicated that acidic oligosaccharides of bovine brain RNase contain both the α-Sia-(2→3)- and -(2→6)-D-Gal linkages. This assumption was further confirmed by the methylation analysis which will be described later. The sialic acid groups released from the intact RNase by *A. ureafaciens* sialidase digestion were all *N*-acetylneuraminic acid and no *N*-glycolylneuraminic acid was detected (data not shown).

Fractionation of the neutral oligosaccharides by Bio-Gel P-4 column chromatography. — Fractions N, A1N, and A2N were subjected to Bio-Gel P-4 column chromatography. As shown in Fig. 2A, Fraction N was separated into two radioactive peaks (I and II) in a molar ratio of 1:3. Fractions A1N and A2N were

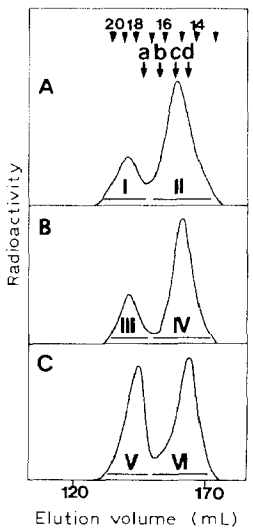


Fig. 2. Bio-Gel P-4 column chromatograms of the radioactive oligosaccharides: (A) Fraction N, (B) Fraction A1N and (C), Fraction A2N. The black arrow-heads indicate the elution positions of D-glucose oligomers (numbers indicate the glucose units) added to the radioactively-labeled samples as internal standards. The black arrows indicate the elution positions of authentic oligosaccharides: (a) $Gal_3 \rightarrow GlcNAc_3 \rightarrow Man_3 \rightarrow GlcNAc \rightarrow Fuc \rightarrow GlcNAc_{OT}$-I, (b) $Gal_2 \rightarrow Gal_2 \rightarrow GlcNAc_2 \rightarrow Man_3 \rightarrow GlcNAc \rightarrow Fuc \rightarrow GlcNAc_{OT}$, (c) $Gal \rightarrow Gal_2 \rightarrow GlcNAc_2 \rightarrow Man_3 \rightarrow GlcNAc \rightarrow Fuc \rightarrow GlcNAc_{OT}$, and (d) $Gal_2 \rightarrow GlcNAc_2 \rightarrow Man_3 \rightarrow GlcNAc \rightarrow Fuc \rightarrow GlcNAc_{OT}$.

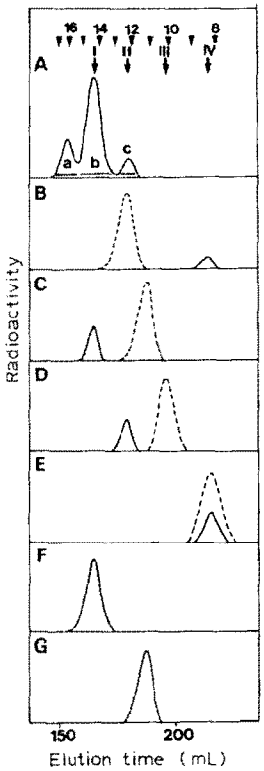


Fig. 3. Sequential exoglycosidase digestion of radioactively-labeled Fraction II. Analytical conditions and black arrow-heads are the same as in Fig. 2. The black arrows indicate the elution positions of authentic oligosaccharides: (I) $Gal_2 \rightarrow GlcNAc_2 \rightarrow Man_3 \rightarrow GlcNAc \rightarrow Fuc \rightarrow GlcNAc_{OT}$, (II) $GlcNAc_2 \rightarrow Man_3 \rightarrow GlcNAc \rightarrow Fuc \rightarrow GlcNAc_{OT}$-I, (III) $GlcNAc \rightarrow Man_3 \rightarrow GlcNAc \rightarrow Fuc \rightarrow GlcNAc_{OT}$, and (IV) $Man_3 \rightarrow GlcNAc \rightarrow Fuc \rightarrow GlcNAc_{OT}$. (A) Radioactively-labeled Fraction II after incubation with diplococcal β-D-galactosidase; (B) (····) and (——) indicate the elution patterns of Oligosaccharide *b* and Oligosaccharide *c* in (A) after digestion by diplococcal *N*-acetyl-β-D-hexosaminidase, respectively; (C) (——) and (····) indicate the elution profiles of Oligosaccharide *a* in (A) and the dotted-line component in (B) incubated with coffee bean α-D-galactosidase, respectively; (D) (——) and (····) indicate the solid-line component and the dotted-line component in (C) incubated with diplococcal β-D-galactosidase, respectively; (E) (——) and (····) indicate the solid-line component and the dotted-line component in (D) incubated with diplococcal *N*-acetyl-β-D-hexosaminidase, respectively; (F) Fraction II in Fig. 2A after coffee bean α-D-galactosidase digestion; and (G) the dotted-line component in (B) after incubation with *A. saitoi* α-mannosidase II.

also separated into two peaks (III, IV; and V, VI; respectively) in a molar ratio of 1:2 and 4:3, respectively (Figs. 2B and C).

Structural study of oligosaccharides in Fraction N. — In order to determine the anomeric configuration and sequence of each monosaccharide of oligosaccharides in Fractions I and II, radioactively-labeled Fractions I and II were subjected to sequential exoglycosidase digestion and analyzed by Bio-Gel P-4 column chromatography.

When Fraction II was incubated with diplococcal β-D-galactosidase, which

cleaves the β-D-Gal-(1→4)-D-GlcNAc linkage only[19], 64% of the radioactive component was converted into Oligosaccharide *b* with release of one galactose residue, 8% to Oligosaccharide *c* with release of two galactose groups, and the remaining 28% (Oligosaccharide *a*) was resistant to the enzyme digestion (Fig. 3A).

When Oligosaccharide *c* was incubated with diplococcal *N*-acetyl-β-D-hexosaminidase, which cleaves the β-D-GlcNAc-(1→2)-D-Man linkage, but not the β-D-GlcNAc-(1→4)- and β-D-GlcNAc-(1→6)-D-Man linkages[20], two 2-acetamido-2-deoxy-D-glucosyl groups were released to give a radioactive oligosaccharide having the same mobility as authentic Man_3→GlcNAc→Fuc→$GlcNAc_{OT}$ (Fig. 3B, solid line). The radioactively-labeled product (solid line in Fig. 3B) was converted into radioactive 2-acetamido-2-deoxyglucitol by sequential treatment with jack bean α-D-mannosidase, snail β-D-mannosidase, jack bean *N*-acetyl-β-D-hexosaminidase, and bovine epididymal α-L-fucosidase with release of two mannose, one mannose, one 2-acetamido-2-deoxyglucose, and one fucose unit, respectively (data not shown). On the other hand, Oligosaccharide *b* released one 2-acetamido-2-deoxyglucosyl group by diplococcal *N*-acetyl-β-D-hexosaminidase treatment (dotted line in Fig. 3B). Oligosaccharide *a*, which was resistant to the β-D-galactosidase treatment, was also resistant to the *N*-acetyl-β-D-hexosaminidase digestion (data not shown).

When Oligosaccharide *a* was incubated with coffee bean α-D-galactosidase, two galactosyl groups were released to give a radioactive oligosaccharide having the same mobility as authentic Gal_2→$GlcNAc_2$→Man_3→GlcNAc→Fuc→$GlcNAc_{OT}$ (Fig. 3C, solid line). The radioactively-labeled product (solid line in Fig. 3C) released two galactosyl groups by incubation with diplococcal β-D-galactosidase (Fig. 3D, solid line), and then two 2-acetamido-2-deoxyglucosyl groups by subsequent diplococcal *N*-acetyl-β-D-hexosaminidase digestion (Fig. 3E, solid line). The mobility of the radioactively-labeled peak in Fig. 3E was the same as that of authentic Man_3→GlcNAc→Fuc→$GlcNAc_{OT}$. The radioactively-labeled product (dotted line in Fig. 3B) released one galactosyl group by incubation with coffee bean α-D-galactosidase (dotted line in Fig. 3C). The radioactively-labeled product was converted into a radioactively-labeled oligosaccharide having the same mobility as that of authentic Man_3→GlcNAc→Fuc→$GlcNAc_{OT}$ by sequential digestion with diplococcal β-D-galactosidase (dotted line in Fig. 3D) and diplococcal *N*-acetyl-β-D-hexosaminidase (Fig. 3E, dotted line). One each of galactose and 2-acetamido-2-deoxyglucose residues were released by this treatment.

When Fraction II was incubated with coffee bean α-D-galactosidase, a slim, radioactive peak having the same mobility as authentic Gal_2→$GlcNAc_2$→Man_3→GlcNAc→Fuc→$GlcNAc_{OT}$ was detected (Fig. 3F). Digestion of this radioactively-labeled product with diplococcal β-D-galactosidase and diplococcal *N*-acetyl-β-D-hexosaminidase gave exactly the same results, as shown by the solid lines in Fig. 3D and E, respectively. The results so far described indicated that Fraction II was a mixture of biantennary, complex-type oligosaccharides having different proportions of α-Gal-(1→?)-β-Gal-(1→4)-β-GlcNAc-(1→ and β-Gal-(1→4)-β-GlcNAc-(1→ outer chains.

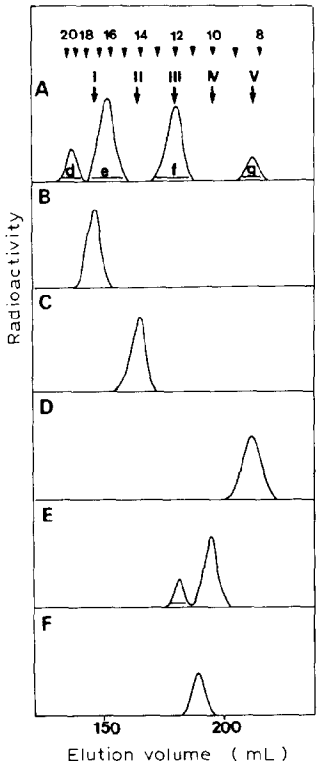
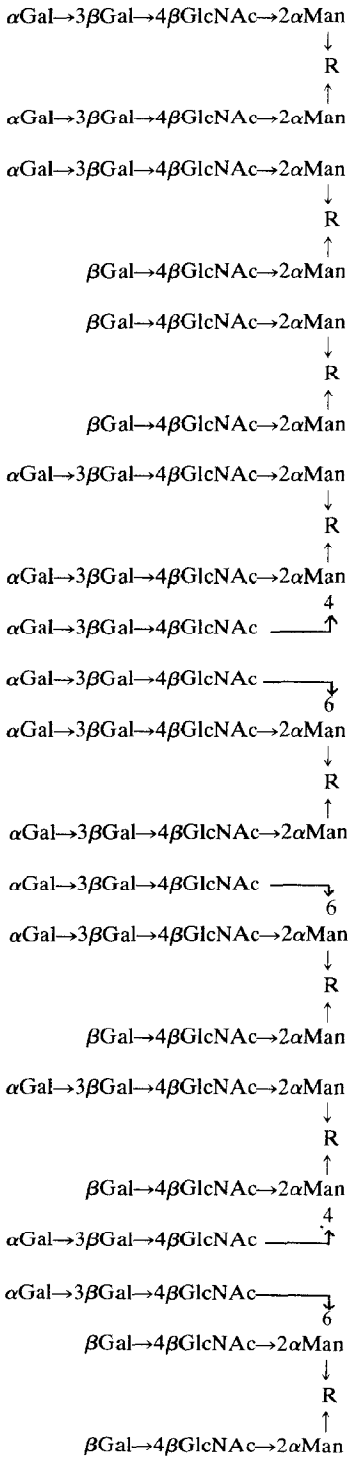
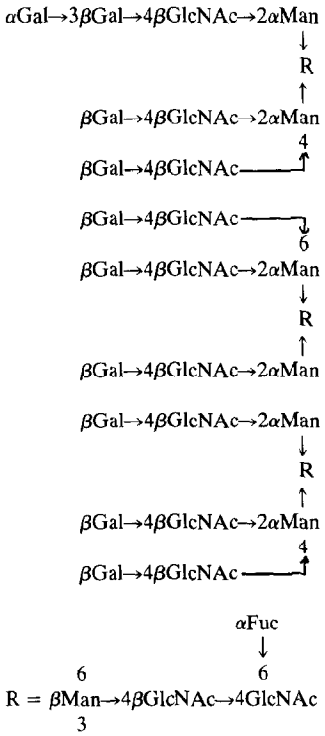


Fig. 4. Sequential exoglycosidase digestion of radioactively-labeled Fraction I. The black arrow-heads are the same as in Fig. 2. The black arrows indicate the elution positions of authentic oligosaccharides: (I) $Gal_3 \rightarrow GlcNAc_3 \rightarrow Man_3 \rightarrow GlcNAc \rightarrow Fuc \rightarrow GlcNAc_{OT}$-I, (II) $GlcNAc_3 \rightarrow Man_3 \rightarrow GlcNAc \rightarrow Fuc \rightarrow GlcNAc_{OT}$, (III) $GlcNAc_2 \rightarrow Man_3 \rightarrow GlcNAc \rightarrow Fuc \rightarrow GlcNAc_{OT}$-II, (IV) $GlcNAc \rightarrow Man_3 \rightarrow GlcNAc \rightarrow Fuc \rightarrow GlcNAc_{OT}$, and (V) $Man_3 \rightarrow GlcNAc \rightarrow Fuc \rightarrow GlcNAc_{OT}$. (A) Radioactively-labeled Fraction I after incubation with a mixture of jack bean α-D-galactosidase and *N*-acetyl-β-D-hexosaminidase; (B) radioactively-labeled Fraction I after incubation with coffee bean α-D-galactosidase; (C) radioactive peak in (B) after diplococcal β-D-galactosidase digestion; (D) radioactive peak in (C) after jack bean *N*-acetyl-β-D-hexosaminidase digestion; (E) radioactively-labeled peak in (C) after diplococcal *N*-acetyl-β-D-hexosaminidase digestion; and (F) the minor component as indicated by a bar in (E) after *A. saitoi* α-mannosidase II digestion.

Because Oligosaccharide *a* (Fig. 3) was not hydrolyzed by diplococcal β-D-galactosidase, a structure of biantennary sugar chains with two trisaccharide outer chains was attributed to it. Thus, Oligosaccharide *c* (Fig. 3) would derive from the biantennary sugar chains having two disaccharide outer chains. In contrast, Oligosaccharide *b* was considered to be derived from the biantennary sugar chains having one of each di- and tri-saccharide outer chains by removal of one β-Gal-(1$\rightarrow$ group. Therefore, the dotted line peak in Fig. 3B corresponds to a compound having the structure, α-Gal-(1$\rightarrow$)-β-Gal-(1$\rightarrow$4)-β-GlcNAc-(1$\rightarrow$2)-α-Man-(1$\rightarrow$6 or 3)-[α-Man-(1$\rightarrow$3 or 6)]-β-Man-(1$\rightarrow$4)-β-GlcNAc-(1$\rightarrow$4)-[α-Fuc-(1$\rightarrow$6)]-$GlcNAc_{OT}$. When the radioactively-labeled product (dotted line in Fig. 3B) was treated with *A. saitoi*



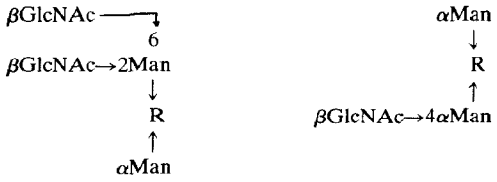
αGal→3βGal→4βGlcNAc→2αMan ↓ R ↑ αGal→3βGal→4βGlcNAc→2αMan	**1**	2.4 (2.4, 0)
αGal→3βGal→4βGlcNAc→2αMan ↓ R ↑ βGal→4βGlcNAc→2αMan	**2**	24.7 (5.5, 19.2)
βGal→4βGlcNAc→2αMan ↓ R ↑ βGal→4βGlcNAc→2αMan	**3**	28.3 (0.7, 27.6)
αGal→3βGal→4βGlcNAc→2αMan ↓ R ↑ αGal→3βGal→4βGlcNAc→2αMan 4 αGal→3βGal→4βGlcNAc ——↑	**4**	0.3 (0.3, 0)
αGal→3βGal→4βGlcNAc ——↓ 6 αGal→3βGal→4βGlcNAc→2αMan ↓ R ↑ αGal→3βGal→4βGlcNAc→2αMan	**5**	0.1 (0.1, 0)
αGal→3βGal→4βGlcNAc ——↓ 6 αGal→3βGal→4βGlcNAc→2αMan ↓ R ↑ βGal→4βGlcNAc→2αMan	**6**	2.0 (0.4, 1.6)
αGal→3βGal→4βGlcNAc→2αMan ↓ R ↑ βGal→4βGlcNAc→2αMan 4 αGal→3βGal→4βGlcNAc ——↑	**7**	4.8 (0.8, 4.0)
αGal→3βGal→4βGlcNAc——↓ 6 βGal→4βGlcNAc→2αMan ↓ R ↑ βGal→4βGlcNAc→2αMan	**8**	8.8 (0.3, 8.5)



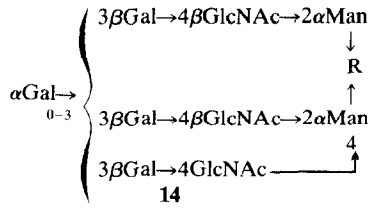
Scheme 1. Proposed structures of the asparagine-linked sugar chains of bovine brain RNase. The numbers at the right indicate the percent molar ratio of each oligosaccharide to total sugar chains. Numbers at the left and right in the parenthesis indicate the percent molar ratio of each oligosaccharide detected in Fractions N and AN to total sugar chains. The D configuration (L configuration for fucose), pyranose ring, and glycosyl linkage at C-1 are assumed for all sugar residues.

α-D-mannosidase II, which removes[9] an α-D-mannosyl group from β-Gal-(1→4)-β-GlcNAc-(1→2)-α-Man-(1→6)-[α-Man-(1→3)]-β-Man-(1→4)-β-GlcNAc-(1→ but not from α-Man-(1→6)-[β-Gal-(1→4)-β-GlcNAc-(1→2)-α-Man-(1→3)]-β-Man-(1→4)-β-GlcNAc-(1→, one mannosyl group was released (Fig. 3G). This result indicated that the α-Gal-(1→)-β-Gal-(1→4)-β-GlcNAc-(1→ group in the outer chain of Oligosaccharide *b* is exclusively linked to the α-Man-(1→6) branch. These results, together with the methylation data which will be described later, indicated that Fraction II contains oligosaccharides **1, 2,** and **3** in the molar ratio of 7:16:2.

When digested with a mixture of jack bean β-D-galactosidase and *N*-acetyl-β-D-hexosaminidase, four radioactive oligosaccharides (Oligosaccharides *d, e, f,* and *g*, Fig. 4A) were obtained from Fraction I. On the other hand, when Fraction I was first incubated with coffee bean α-D-galactosidase, it was converted into a product showing a slim peak having the same elution position as authentic Gal_3→GlcNAc→Man_3→GlcNAc→Fuc→$GlcNAc_{OT}$-I (Fig. 4B). When digested with diplo-



12 13



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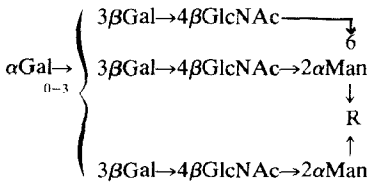

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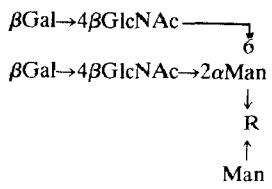


16

coccal β-D-galactosidase, the radioactively-labeled product (Fig. 4B) released three galactosyl groups (Fig. 4C). The product in the radioactive peak was then converted, by jack bean *N*-acetyl-β-D-hexosaminidase digestion, into a radioactively-labeled component having the same mobility as authentic Man_3→GlcNAc→Fuc→$GlcNAc_{OT}$ (Fig. 4D). These results indicated that Fraction I is a mixture of triantennary, complex-type oligosaccharides that contain three, two, one, or none α-Gal-(1→)-β-Gal-(1→4)-β-GlcNAc-(1→ groups in their outer chains.

The product of the radioactively-labeled peak in Fig. 4C was converted into a mixture of two components that migrated as 11.6 and 10.0 glucose units, respectively, by digestion with diplococcal *N*-acetyl-β-D-hexosaminidase (Fig. 4E). The molar ratio of the two components was 29:71. The minor component shown in Fig. 4E released one mannosyl group by *A. saitoi* α-D-mannosidase II digestion (Fig. 4F), but the major component was resistant to this enzyme (data not shown). It is known[20] that diplococcal *N*-acetyl-β-D-hexosaminidase hydrolyzes the β-GlcNAc-(1→2)-D-Man linkage in the β-GlcNAc-(1→4)-[β-GlcNAc-(1→2)]-Man group but not in the β-GlcNAc-(1→6)-[β-GlcNAc-(1→2)]-Man group. Based on the substrate specificities of diplococcal *N*-acetyl-β-D-hexosaminidase and *A. saitoi* α-mannosid-

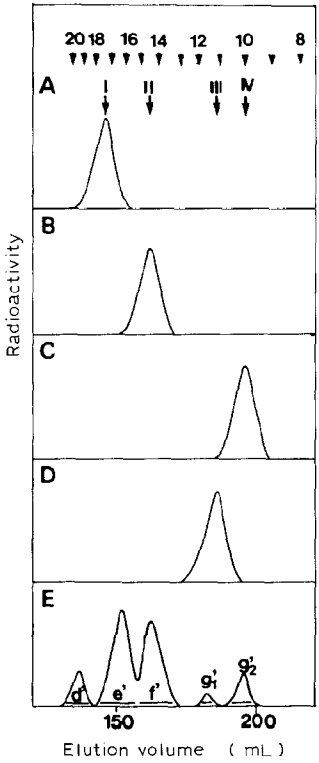


Fig. 5. Exoglycosidase digestion of radioactive oligosaccharides. The black arrow-heads are the same as in Fig. 2. The black arrows indicate the elution positions of authentic oligosaccharides: (I) $Gal_3 \rightarrow GlcNAc_3 \rightarrow Man_3 \rightarrow GlcNAc \rightarrow Fuc \rightarrow GlcNAc_{OT}$-I, (II) $Gal_2 \rightarrow GlcNAc_2 \rightarrow Man_3 \rightarrow GlcNAc \rightarrow Fuc \rightarrow GlcNAc_{OT}$, (III) $Gal \rightarrow GlcNAc \rightarrow Man_3 \rightarrow GlcNAc \rightarrow Fuc \rightarrow GlcNAc_{OT}$, and (IV) $GlcNAc \rightarrow Man_3 \rightarrow GlcNAc \rightarrow Fuc \rightarrow GlcNAc_{OT}$. (A) Oligosaccharide *d* in Fig. 4A after incubation with coffee bean α-D-galactosidase; (B) Oligosaccharide *e* in Fig. 4A after incubation with coffee bean α-D-galactosidase; (C) Oligosaccharide *e-1* (the DSA-unbound fraction of the peak in Fig. 5B; for detail, see text) after sequential incubation with diplococcal β-D-galactosidase and diplococcal *N*-acetyl-β-D-hexosaminidase; (D) Oligosaccharide *f* in Fig. 4A after incubation with coffee bean α-D-galactosidase; and (E) radioactively-labeled Fraction I in Fig 2A after incubation with a mixture of diplococcal β-D-galactosidase and diplococcal *N*-acetyl-β-D-hexosaminidase.

ase II, the peak at 11.6 glucose units was considered to contain oligosaccharide **12**, and the peak at 10.0 glucose units heptasaccharide **13**. These results indicated that Fraction I was a mixture of oligosaccharides **14** and **15**.

In order to determine the number and the location of α-galactosyl groups, Oligosaccharides *d, e, f,* and *g* were subjected to further exoglycosidase digestion and lectin column chromatography. When Oligosaccharide *d* was digested with coffee bean α-D-galactosidase, it was converted into a radioactive oligosaccharide having the same mobility as authentic $Gal_3 \rightarrow GlcNAc_3 \rightarrow Man_3 \rightarrow GlcNAc \rightarrow Fuc \rightarrow GlcNAc_{OT}$-I (Fig. 5A). The difference in size between Oligosaccharide *d* and the product of its enzymic digestion was 2.7 glucose units, indicating that three galacto-

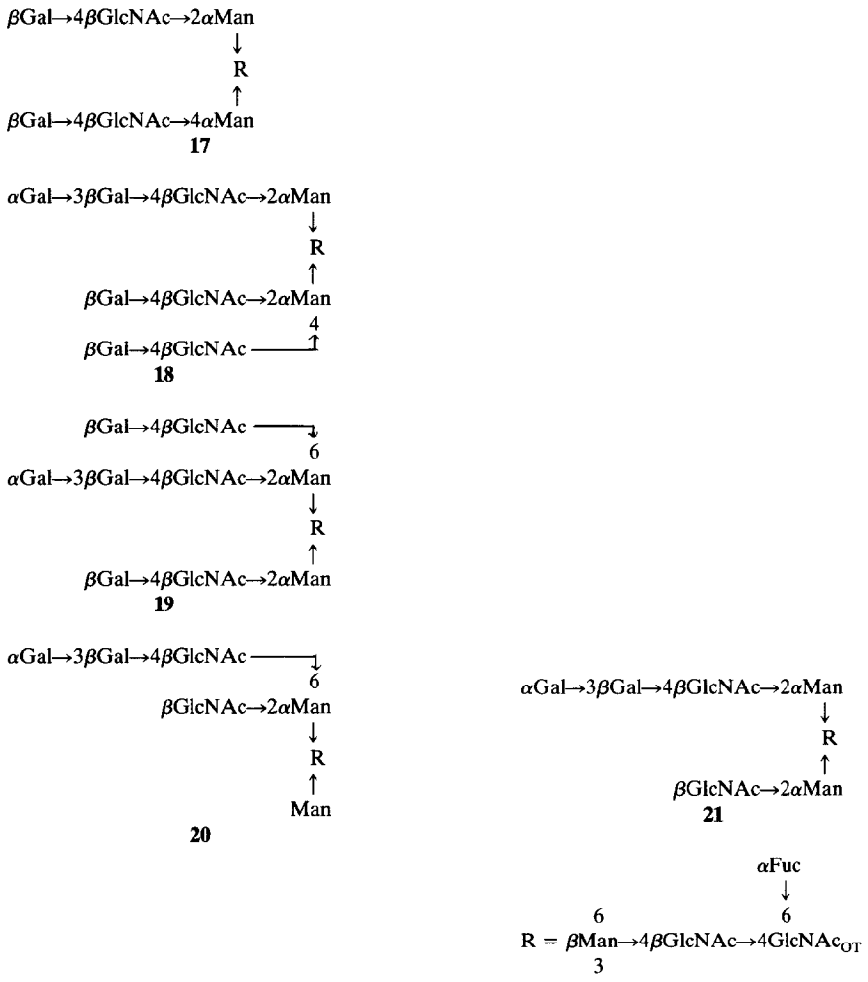
syl groups had been released. The elution profiles of its sequential exoglycosidase digestion products with diplococcal β-D-galactosidase and jack bean *N*-acetyl-β-D-hexosaminidase were the same as those shown in Figs. 4C and D, respectively. These results indicated that Oligosaccharide *d* is a triantennary, complex-type oligosaccharide containing three α-galactosyl groups at the nonreducing ends.

When the product corresponding to the peak in Fig. 5A was treated with a mixture of diplococcal β-D-galactosidase and *N*-acetyl-β-D-hexosaminidase, two products having mobilities of 11.6 and 10.0 glucose units were obtained in a molar ratio of 1:3 (data not shown). Further sequential exoglycosidase digestion revealed that the higher mol.wt. component was β-GlcNAc-(1→6)-[β-GlcNAc-(1→2)]-α-Man-(1→6)-[α-Man-(1→3)]-β-Man-(1→4)-β-GlcNAc-(1→4)-[α-Fuc-(1→6)]-GlcNAc$_{OT}$ and the lower mol. wt. component was α-Man-(1→6)-[β-GlcNAc-(1→4)-α-Man-(1→3)]-β-Man-(1→4)-β-GlcNAc-(1→4)-[α-Fuc-(1→6)]-GlcNAc$_{OT}$ (data not shown). Therefore, Oligosaccharide *d* is considered to be derived from oligosaccharides **4** and **5**.

When Oligosaccharide *e* was digested with coffee bean α-D-galactosidase, it was converted into a radioactively-labeled oligosaccharide having the same mobility as authentic Gal$_2$→GlcNAc$_2$→Man$_3$→GlcNAc→Fuc→GlcNAc$_{OT}$ with release of two galactosyl groups (Fig. 5B). The radioactive products shown in Fig. 5B were converted into Man$_3$→GlcNAc→Fuc→GlcNAc$_{OT}$ by the release of two galactosyl and two 2-acetamido-2-deoxyglucosyl groups by sequential digestion with diplococcal β-D-galactosidase and jack bean *N*-acetyl-β-D-hexosaminidase (data not shown). This result indicated that the peak contains oligosaccharides having the structure, [β-Gal-(1→4)-β-GlcNAc-(1→]$_2${α-Man-(1→6)-[α-Man-(1→3)]-β-Man-(1→4)-β-GlcNAc-(1→4)-[α-Fuc-(1→6)]-GlcNAc$_{OT}$}. When the radioactively-labeled product (Fig. 5B) was subjected to affinity chromatography on a *Datura stramonium* agglutinin (DSA)-Sepharose column, two fractions, one unbound and one bound, were obtained in a molar ratio of 2:1 (data not shown). These fractions were named *e-1* and *e-2*, respectively. The carbohydrate-binding specificity of DSA has been already elucidated by Yamashita *et al.*[11] as follows. All oligosaccharides containing either the β-Gal-(1→4)-β-GlcNAc-(1→6)-[β-Gal-(1→4)-β-GlcNAc-(1→2)]-Man group or the β-Gal-(1→4)-β-GlcNAc-(1→3)-β-Gal-(1→4)-β-GlcNAc-(1→ group in nonsubstituted form are bound to the column and eluted with buffer containing 2-acetamido-2-deoxy-β-D-glucose oligomers. Oligosaccharides having the nonsubstituted β-Gal-(1→4)-β-GlcNAc-(1→4)-[β-Gal-(1→4)-β-GlcNAc-(1→2)]-Man group are retarded in the column. Oligosaccharides that contain none of the groups described above pass through the column without interaction. When the degalactosylated product, obtained from Oligosaccharide *e-2* by diplococcal β-D-galactosidase treatment, was incubated with *A. saitoi* α-mannosidase II, one mannose residue was released (data not shown). The binding specificity of the DSA-Sepharose column and the substrate specificities of diplococcal β-D-galactosidase[19] and *A. saitoi* α-mannosidase II[9] suggested for Oligosaccharide *e-2* structure **16** which derives from oligosaccharide **6**.

Oligosaccharide *e-1* was converted into a radioactive oligosaccharide having the same mobility as authentic GlcNAc→Man_3→GlcNAc→Fuc→$GlcNAc_{OT}$ upon sequential incubation with diplococcal β-D-galactosidase and diplococcal *N*-acetyl-β-D-hexosaminidase (Fig. 5C) with the release of two galactose and one 2-acetamido-2-deoxyglucose units. This radioactive oligosaccharide was resistant to digestion with *A. saitoi* α-D-mannosidase II (data not shown). These results indicated structure **17** for Oligosaccharide *e-1*, which derives from oligosaccharide **7**.

When Oligosaccharide *f* was digested with coffee bean α-D-galactosidase, it was converted into a radioactive oligosaccharide having the same mobility as authentic Gal→GlcNAc→Man_3→GlcNAc→Fuc→$GlcNAc_{OT}$ (Fig. 5D). Approxi-



Scheme 2. See legend to Scheme 1 for configuration, ring, size, and location of glycosyl linkage of sugar residues.

mately 30% of the radioactive oligosaccharide passed through a concanavalin A (Con A)-Sepharose column, while the remainder bound to the column and was eluted with 0.1M methyl α-D-mannopyranoside solution (data not shown). These two fractions were named, *f-1* and *f-2*, respectively. As reported earlier[10], complex-type oligosaccharides, in which the two α-D-mannosyl residues occur either as non-reducing terminal groups or substituted only at C-2 with outer chains, bind to a Con A-Sepharose column and are eluted with a buffer containing methyl α-D-mannopyranoside. Therefore, the structure of oligosaccharide *f-1* is α-Man-(1→6)-[β-Gal-(1→4)-β-GlcNAc-(1→4)-α-Man-(1→3)]-β-Man-(1→4)-β-GlcNAc-(1→4)-[α-Fuc-(1→6)]-$GlcNAc_{OT}$ or β-Gal-(1→4)-β-GlcNAc-(1→6)-α-Man-(1→6)-[α-Man-(1→3)]-β-Man-(1→4)-β-GlcNAc-(1→4)-[α-Fuc-(1→6)]-$GlcNAc_{OT}$, or both. When Oligosaccharide *f-1* was incubated with *A. saitoi* α-D-mannosidase II, it released one mannose residue (data not shown). These results indicated that the structure of Oligosaccharide *f-1* is the last named and that the octasaccharide derived from oligosaccharide **8**.

Oligosaccharide *f-2* released also one mannose unit by *A. saitoi* α-D-mannosidase II treatment (data no shown). This result indicated a structure β-Gal-(1→4)-β-GlcNAc-(1→2)-α-Man-(1→6)-[α-Man-(1→3)]-β-Man-(1→4)-β-GlcNAc-(1→4)-[α-Fuc-(1→6)]-$GlcNAc_{OT}$ and that Oligosaccharide *f-2* was derived from compound **18** or **19**, or both. In order to determine the ratio of the two possible oligosaccharides, radioactively-labeled Fraction I was digested with a mixture of diplococcal β-D-galactosidase and *N*-acetyl-β-D-hexosaminidase. Upon Bio-Gel P-4 column chromatography, five radioactive components were deteted (Fig. 5E). Coffee bean α-D-galactosidase treatment revealed that Oligosaccharides *d'*, *e'*, and *f'* contain three, two, and one α-D-galactosyl groups, and Oligosaccharides *g1'* and *g2'* do not contain any α-D-galactosyl group (data not shown). Oligosaccharides *d'* and *e'* gave the same results as Oligosaccharides *d* and *e* when subjected to sequential exoglycosidase digestion. When degalactosylated Oligosaccharide *f'*, which was obtained by treatment with coffee bean α-D-galactosidase and diplococcal β-D-galactosidase, was digested with *A. saitoi* α-mannosidase II, only 30% of it released one mannose unit (data not shown). This result indicated that Oligosaccharide *f'* was a mixture of the two isomeric oligosaccharides **20** and **21**, in a molar ratio of 3:7. The former was converted into Oligosaccharide *f-1* and the latter into Oligosaccharide *f-2* by coffee bean α-D-galactosidase and jack bean *N*-acetyl-β-D-hexosaminidase treatment. Therefore, Oligosaccharide *f-2* was exclusively derived from oligosaccharide **9**.

Oligosaccharides *g1'* and *g2'* were converted into α-Man-(1→6)-[α-Man-(1→3)]-β-Man-(1→4)-β-GlcNAc-(1→4)-[α-Fuc-(1→6)]-$GlcNAc_{OT}$ by jack bean *N*-acetyl-β-D-hexosaminidase digestion with release of two and one 2-acetamido-2-deoxyglucose units, respectively (data not shown). Since these units were not released by diplococcal *N*-acetyl-β-D-hexosaminidase, the structure of Oligosaccharide *g1'* is β-GlcNAc-(1→6)-[β-GlcNAc-(1→2)]-α-Man-(1→6)-[α-Man-(1→3)]-β-Man-(1→4)-β-GlcNAc-(1→4)-[α-Fuc-(1→6)]-$GlcNAc_{OT}$ and that of Oligosacchar-

TABLE I

METHYLATION ANALYSIS OF OLIGOSACCHARIDE FRACTIONS

Partially O-methylated sugars	*Fractions (molar ratio)*[a]			
	N	*A*	*AN*	*N + AN*[b]
Fucitol				
2,3,4-Tri-*O*-methyl (1,5-di-*O*-acetyl)	0.8	0.8	0.8	0.8
Galactitol				
2,3,4,6-Tetra-*O*-methyl (1,5-di-*O*-acetyl)	2.2	0.9	2.4	2.4
2,4,6-Tri-*O*-methyl (1,3,5-tri-*O*-acetyl)	1.3	1.3	0.6	
2,3,4-Tri-*O*-methyl (1,5,6-tri-*O*-acetyl)		0.9		
Mannitol				
3,4,6-Tri-*O*-methyl (1,2,5-tri-*O*-acetyl)	1.8	1.6	1.6	1.5
3,6-Di-*O*-methyl (1,2,4,5-tetra-*O*-acetyl)	0.2	0.2	0.3	0.3
3,4-Di-*O*-methyl (1,2,5,6-tetra-*O*-acetyl)	[c]	[c]	[c]	[c]
2,4-Di-*O*-methyl (1,3,5,6-tetra-*O*-acetyl)	1.0	1.0	1.0	1.0
2-N-Methylacetamido-2-deoxyglucitol				
1,3,5-Tri-*O*-methyl (4,6-di-*O*-acetyl)	0.9	0.8	0.9	0.9
3,6-Di-*O*-methyl (1,4,5-tri-*O*-acetyl)	3.2	3.0	3.2	3.3

[a]The values were estimated by taking the value of 2,4-di-*O*-methylmannitol as 1.0. [b]Exhaustively digested with sialidase and then with coffee bean α-D-galactosidase and freed from sialic acid and galactose by paper chromatography (for detail, see the Experimental section). [c]Less than 0.1.

ide *g2'* is α-Man-(1→6)-[β-GlcNAc-(1→4)-α-Man-(1→3)]-β-Man-(1→4)-β-GlcNAc-(1→4)-[α-Fuc-(1→6)]-$GlcNAc_{OT}$. Therefore, Oligosaccharides *g1'* and *g2'* derived from oligosaccharides **10**, and **11**, respectively. Oligosaccharide *g*, which was eluted at the same position as that of authentic Man_3→GlcNAc→Fuc→$GlcNAc_{OT}$, derived from these two dodecasaccharides.

Structures of oligosaccharides from Fraction A1N. — When Fraction IV was incubated with diplococcal β-D-galactosidase, 79% of the fraction was converted into Oligosaccharide *b* (Fig. 3A) with release of one galactose unit, and the remainder was converted into Oligosaccharide *c* (Fig. 3A) with release of two galactose units (data not shown). Structural assignment of these oligosaccharides were performed as described in the previous section. The results indicated that Fraction IV was a mixture of biantennary, complex-type oligosaccharides with and without a nonreducing, terminal α-D-galactosyl group.

When Fraction III was incubated with a mixture of jack bean β-D-galactosidase and *N*-acetyl-β-D-hexosaminidase, it was converted into a mixture of radioactive Oligosaccharides *e, f,* and *g* (Fig. 4A) (other data not shown). Structural assignment of these three oligosaccharides were performed as previously described. The results indicated that Fraction III contained triantennary, complex-type oligosaccharides having zero, one, and two nonreducing terminal α-D-galacto-

syl groups. The restricted distribution of α-D-galactosyl groups in oligosaccharides **6–9** was confirmed by the study of Fraction N.

Structures of oligosaccharides of Fraction A2N. — When Fraction VI was incubated with diplococcal β-D-galactosidase, it was converted into radioactive Oligosaccharide *c* (Fig. 3A) with release of two galactose units (other data not shown). The structure of the radioactive oligosaccharide was confirmed by a series of analyses previously described. The results indicated that Fraction VI contained only biantennary, complex-type oligosaccharides devoid of α-D-galactosyl groups.

When Fraction V was incubated with a mixture of jack bean β-D-galactosidase and *N*-acetyl-β-D-hexosaminidase, 79% of the radioactive oligosaccharide was converted into the radioactive Oligosaccharide *f* and the remainder into Oligosaccharide *g* (Fig. 4A, other data not shown). The results indicated that Fraction V contained triantennary, complex-type oligosaccharides with or without one α-D-galactosyl nonreducing, terminal group. The structures of oligosaccharides having one α-D-galactosyl residue as in **8** and **9** were confirmed by the study of Fraction N.

Methylation analyses. — The analytical data so far described indicate that bovine brain RNase contains a great variety of sugar chains. Since it was practically impossible to obtain methylation data for each individual oligosaccharide, the study of the structure of each oligosaccharide was performed by sequential exoglycosidase digestion as described already. However, knowledge of the sugar linkages was indispensable for the rational interpretation of the data. Therefore, methylation analysis of Fractions N, AN, (A1N plus A2N), and A (A1 plus A2) was performed and the data are summarized in Table I. Complete absence of 2-mono-*O*-methylmannitol indicated that no "bisecting" 2-acetamido-2-deoxyglucosyl residue was present in the oligosaccharides. Therefore, the amount of 2,4-di-*O*-methylmannitol was taken as 1.0 in order to calculate the molar ratio of each methylated monosaccharide. Detection of approximately one mole of 1,3,5-tri-*O*-methyl-2-*N*-methylacetamido-2-deoxyglucitol and no 1,3,5,6-tetra-*O*-methyl-2-*N*-methylacetamido-2-deoxyglucitol per mol of oligosaccharide indicated that all oligosaccharides in these three fractions contain the fucosylated trimannosyl core, α-Man-(1→6)-[α-Man-(1→3)]-β-Man-(1→4)-β-GlcNAc-(1→4)-[α-Fuc-(1→6)]-$GlcNAc_{OT}$. When the data of Fraction A were compared with those of Fraction AN, the amount of 2,4,6-tri-*O*-methylgalactitol was significantly decreased and that of 2,3,4-tri-*O*-methylgalactitol totally vanished after sialidase treatment. In compensation for the decrease of these galactose derivatives, the amount of 2,3,4,6-tetra-*O*-methylgalactitol increased. These results indicated that *N*-acetylneuraminic acid is linked to C-6 and C-3 of the nonreducing, subterminal galactosyl residues of oligosaccharides in Fraction A. This is consistent with the results of NDV sialidase digestion described in the previous section, *i.e.*, that the acidic oligosaccharides in both Fractions A1 and A2 contain two types of sialic acid linkages, α-Neu5Ac-(2→3)-Gal and α-Neu5Ac-(2→6)-Gal. Detection of only 3,6-di-*O*-methyl-2-*N*-methylacetamido-2-deoxyglucitol besides the 1,3,5-tri-*O*-methyl derivative indicated that all 2-acetamido-2-deoxyglucosyl residues in the sugar chains are substituted only at C-4. In Fractions N and

AN, 2,3,4-tri-*O*-methylfucitol and 2,3,4,6-tetra-*O*-methylgalactitol were detected as corresponding to the nonreducing, terminal sugar groups. Detection of 2,4,6-tri-*O*-methylgalactitol indicated that some of the oligosaccharides in both Fractions N and AN contain galactose residues substituted at C-3. The results of sequential exoglycosidase digestion described earlier, indicated that the galactose residues occur as the α-Gal-(1→3)-β-Gal-(1→ group and, indeed, no 2,4,6-tri-*O*-methylgalactitol was detected in Fractions N and AN after α-D-galactosidase digestion (Table I). Detection of 3,4,6-tri-*O*-methyl-, 3,6-di-*O*-methyl-, and 3,4-di-*O*-methylmannitol indicated that the two α-D-mannosyl residues of the trimannosyl core of oligosaccharides in the three fractions occur as →2)-α-Man-(1→; →2 and 4)-α-Man-(1→; and →2 and →6)-α-Man-(1→ residues.

DISCUSSION

The present data indicated that only one asparagine-linked sugar chain is attached to the peptide chain of bovine brain RNase molecule, but the structure of this sugar chain shows an extraordinarily high heterogeneity. Although all these variations of the sugar chain contain the structure, α-Man-(1→6)-[α-Man-(1→3)]-β-Man-(1→4)-β-GlcNAc-(1→4)-[α-Fuc-(1→6)]-GlcNAc, as the core portion, they are different in the branching and in the outer-chain structure. The majority of the structures (55%) have a biantennary structure, and the remainder have 2,4-branched (32%) and 2,6-branched (13%) triantennary structures. It is noteworthy that no tetraantennary structure was detected despite the presence of the two isomeric, triantennary structures. The heterogeneity of the sugar chain was also due to the occurrence of four different outer chains, namely, β-Gal-(1→4)-GlcNAc, α-Gal-(1→3)-β-Gal-(1→4)-GlcNAc, α-Neu5Ac-(2→3)-β-Gal-(1→4)-GlcNAc, and α-Neu5Ac-(2→6)-β-Gal-(1→4)-GlcNAc.

The α-D-galactosyl group was detected as the nonreducing, terminal residue of the asparagine-linked sugar chains of many glycoproteins, such as mouse Thy-1 antigen[21], mouse laminin[22], bovine complement Clq[13,14], and bovine thyroglobulin[23], as well as calf-thymocyte, plasma-membrane glycoproteins[14,24], and calf-thyroid, cell-surface glycoproteins[25]. Occurrence of the α-Gal-(1→3)-Gal group is species-specific because human thyroglobulin[23] and human complement Clq[26] do not contain this group. Organ-specific distribution of the α-Gal-(1→3)-Gal group was also demonstrated for the bovine species. The sugar chains of many bovine glycoproteins have been determined. These include prothrombin[27], fibronectin[28], blood coagulation factors[29,30] and immunoglobulin G (ref. 31), γ-glutamyltranspeptidases from kidney and liver[32,33], and two glycoproteins located in the eye, rhodopsin[34,35] and retinol-binding protein[36]. However, none of these sugar chains contains the α-Gal-(1→3)-Gal group. These data indicated that the expression of (1→3)-α-D-galactosyltransferase, which is responsible for the formation of the α-Gal-(1→3)-β-Gal-(1→4)-GlcNAc group in the glycoproteins, might be restricted to particular tissues. Species and organ specificities are found in the sugar chain structures of

glycoproteins[33,37]. The specific distribution of (1→3)-α-D-galactosyltransferase indicated that it is one of the enzymes producing such specific structures.

Tri-α-galactosylated triantennary and di-α-galactosylated biantennary structures were found only in the neutral fraction. This is consistent with a previous report that the (1→3)-α-D-galactosyltransferase purified from calf thymus competes with the sialylation of the β-D-galactosyl group in the β-Gal-(1→4)-GlcNAc outer chain by (2→6)- and (2→3)-α-sialyltransferases[38]. Bovine brain sialyltransferase also may not act on the α-Gal-(1→3)-Gal group. The α-Gal-(1→3)-β-Gal-(1→4)-β-GlcNAc-(1→ group is, generally, predominantly linked to the α-Man-(1→6) arm of the sugar chain of bovine brain RNase. This tendency was also found for the sugar chains of the bovine complement Clq and thymocyte glycoproteins[14]. This may suggest another characteristics of the (1→3)-α-D-galactosyltransferase. A similar preferential addition of the sugar residue to the β-Gal-(1→4)-β-GlcNAc-(1→6)-α-Man-(1→6) arm was also observed in the case of the *N*-acetylglucosaminyltransferase that forms the β-GlcNAc-(1→3)-Gal group[39].

The carbohydrate structures of bovine brain RNase are quite different from those of pancreatic RNase B (ref. 4), and probably different from those of kidney RNase K2 on the basis of the analytical data for the monosaccharide composition[40]. Recently, Yamashita *et al.*[1] reported that the glycosylation of RNase of human viscera and body fluids showed organ-specific differences, suggesting the possible use of the enzymes as markers for the diagnosis of the disorders of various organs.

Mono Q anion-exchange column chromatography has recently been used for the separation of sialooligosaccharides[41,42]. In these studies, however, a gradient of sodium chloride was used to separate the acidic oligosaccharides. In the present work, we used a gradient of acetate buffer for the separation of sialooligosaccharides. This buffer is very convenient because desalting can be performed by simply passing the solution through a Dowex 50 (H^+) column and evaporating it to dryness.

REFERENCES

1 K. YAMASHITA, A. HITOI, M. IRIE, AND A. KOBATA, *Arch. Biochem. Biophys.*, 250 (1986) 263–266.
2 T. H. PLUMMER, JR., AND C. H. W. HIRS, *J. Biol. Chem.*, 239 (1964) 2530–2538.
3 T. H. PLUMMER, JR., *J. Biol. Chem.*, 243 (1968) 5961–5966.
4 C.-J. LIANG, K. YAMASHITA, AND A. KOBATA, *J. Biochem. (Tokyo)*, 88 (1980) 51–58.
5 M. ELSON AND D. G. GLITZ, *Biochemistry*, 14 (1975) 1471–1476.
6 Y.-T. LI AND S.-C. LI, *Methods Enzymol.*, 28 (1972) 702–713.
7 L. R. GLASGOW, J. C. PAULSON, AND R. L. HILL, *J. Biol. Chem.*, 252 (1977) 8615–8623.
8 J. C. PAULSON, J. WEINSTEIN, L. DORLAND, H. VAN HALBEEK, AND J. F. G. VLIEGENTHART, *J. Biol. Chem.*, 257 (1982) 12734–12738.
9 J. AMANO AND A. KOBATA, *J. Biochem. (Tokyo)*, 99 (1986) 1645–1654.
10 S. OGATA, T. MURAMATSU, AND A. KOBATA, *J. Biochem. (Tokyo)*, 78 (1975) 687–696.
11 K. YAMASHITA, K. TOTANI, T. OHKURA, S. TAKASAKI, I. J. GOLDSTEIN, AND A. KOBATA, *J. Biol. Chem.*, 262 (1987) 1602–1607.
12 T. OHKURA, T. ISOBE, K. YAMASHITA, AND A. KOBATA, *Biochemistry*, 24 (1984) 503–508.
13 T. MIZUOCHI, T. TANIGUCHI, J. FUJII-KADOWAKI, K. YONEMASU, T. SASAKI, AND A. KOBATA, *J. Biol. Chem.*, 257 (1982) 13300–13305.
14 S. TAKASAKI, T. MIZUOCHI, AND A. KOBATA, *Glycoconjugate J.*, 3 (1986) 321–330.

15 A. HITOI, K. YAMASHITA, Y. NIWATA, M. IRIE, N. KOCHIBE, AND A. KOBATA, *J. Biochem. (Tokyo)*, 101 (1987) 29–41.
16 T. MIZUOCHI, R. NISHIMURA, C. DERAPPE, T. TANIGUCHI, T. HAMAMOTO, M. MOCHIZUKI, AND A. KOBATA, *J. Biol. Chem.*, 258 (1983) 14126–14129.
17 K. YAMASHITA, T. MIZUOCHI, AND A. KOBATA, *Methods Enzymol.*, 83 (1982) 105–126.
18 S. TAKASAKI, T. MIZUOCHI, AND A. KOBATA, *Methods Enzymol.*, 83 (1982) 263–268.
19 J. C. PAULSON, J. P. PRIEELS, L. R. GLASGOW, AND R. L. HILL, *J. Biol. Chem.*, 253 (1978) 5617–5624.
20 K. YAMASHITA, T. OHKURA, H. YOSHIMA, AND A. KOBATA, *Biochem. Biophys. Res. Commun.*, 100 (1981) 226–232.
21 H. VAN HALBEEK, S. R. CARLSSON, AND T. STIGBRAND, *Proc. Int. Symp. Glycoconjugates, VIIIth*, (1985) 118–119.
22 R. G. ARUMUGHAM, T. C.-Y. HSIEH, M. L. TRANZER, AND R. A. LAINE, *Biochim. Biophys. Acta*, 883 (1986) 112–126.
23 R. G. SPIRO AND V. D. BHOYROO, *J. Biol. Chem.*, 259 (1984) 9858–9866.
24 H. YOSHIMA, S. TAKASAKI, AND A. KOBATA, *J. Biol. Chem.*, 255 (1980) 10793–10804.
25 A. S. B. EDGE AND R. G. SPIRO, *J. Biol. Chem.*, 260 (1985) 15332–15338.
26 T. MIZUOCHI, K. YONEMASU, K. YAMASHITA, AND A. KOBATA, *J. Biol. Chem.*, 253 (1978) 7404–7409.
27 T. MIZUOCHI, K. YAMASHITA, K. FUJIKAWA, W. KISIEL, AND A. KOBATA, *J. Biol. Chem.*, 254 (1979) 6419–6425.
28 S. TAKASAKI, K. YAMASHITA, K. SUZUKI, S. IWANAGA, AND A. KOBATA, *J. Biol. Chem.*, 254 (1979) 8548–8553.
29 T. MIZUOCHI, K. YAMASHITA, K. FUJIKAWA, K. TITANI, AND A. KOBATA, *J. Biol. Chem.*, 255 (1980) 3526–3531.
30 T. MIZUOCHI, T. TANIGUCHI, K. FUJIKAWA, K. TITANI, AND A. KOBATA, *J. Biol. Chem.*, 258 (1983) 6020–6024.
31 T. TAI, S. ITO, K. YAMASHITA, T. MURAMATSU, AND A. KOBATA, *Biochem. Biophys. Res. Commun.*, 65 (1975) 968–974.
32 K. YAMASHITA, Y. TACHIBANA, H. SHICHI, AND A. KOBATA, *J. Biochem. (Tokyo)*, 93 (1983) 135–147.
33 A. KOBATA, in V. GINSBURG AND P. W. ROBBINS (Eds.), *Biology of Carbohydrates*, Vol. 2, J. Wiley, New York, 1984, pp. 87–161.
32 C.-J. LIANG, K. YAMASHITA, C. G. MUELLENBERG, H. SHICHI, AND A. KOBATA, *J. Biol. Chem.*, 254 (1979) 6414–6418.
35 M. N. FUKUDA, D. S. PAPERMASTER, AND P. A. HARGRAVE, *J. Biol. Chem.*, 254 (1979) 8201–8207.
36 T. TANIGUCHI, A. J. ADLER, T. MIZUOCHI, N. KOCHIBE, AND A. KOBATA, *J. Biol. Chem.*, 261 (1986) 1730–1736.
37 K. YAMASHITA, A. HITOI, N. TATEISHI, T. HIGASHI, Y. SAKAMOTO, AND A. KOBATA, *Arch. Biochem. Biophys.*, 225 (1983) 993–996.
38 W. M. BLANKEN AND D. H. VAN DEN EIJNDEN, *J. Biol. Chem.*, 260 (1985) 12927–12934.
39 D. H. VAN DEN EIJNDEN, D. H. JOZIASSE, A. H. L. KOENDERMAN, W. M. BLANKEN, W. E. C. M. SCHIPHORST, AND P. L. KOPPEN, *Proc. Int. Symp. Glycoconjugates, VIIIth*, (1985) 285–286.
40 Y. NIWATA, K. OHGI, A. SANDA, Y. TAKIZAWA, AND M. IRIE, *J. Biochem. (Tokyo)*, 97 (1985) 923–934.
41 T. TSUJI AND T. OSAWA, *J. Biochem. (Tokyo)*, 100 (1986) 1387–1398.
42 J. B. L. DAMM, J. P. KAMERLING, G. W. K. VAN DEDEM, AND J. F. G. VLIEGENTHART, *Glycoconjugate J.*, 4 (1987) 129–144.