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STRUCTURE IDENTIFICATION OF THE COMPLEX-TYPE, ASPARAGINE-LINKED SUGAR CHAINS OF β-D-GALACTOSYL-TRANSFERASE PURIFIED FROM HUMAN MILK*†

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ABSTRACT

The asparagine-linked sugar chains of human milk galactosyltransferase were quantitatively released as oligosaccharides from the polypeptide backbone by hydrazinolysis. They were converted into radioactive oligosaccharides by sodium borotritiate reduction after *N*-acetylation, and fractionated by paper electrophoresis and by Bio-Gel P-4 column chromatography after sialidase treatment. Structural studies of each oligosaccharides by sequential exoglycosidase digestion and methylation analysis indicated that the galactosyltransferase contains bi, tri-, and probably tetra-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. Variation is produced by the different locations and numbers of the five different outer chains: β-D-Gal*p*-(1→4)-D-GlcNAc, α-L-Fuc*p*-(1→3)-[β-D-Gal*p*-(1→4)]-D-GlcNAc, α-NeuAc-(2→6)-β-D-Gal*p*-(1→4)-D-GlcNAc, α-L-Fuc*p*-(1→3)-[β-D-Gal*p*-(1→4)]-β-D-Glc*p*NAc-(1→3)-β-D-Gal*p*-(1→4)-[α-L-Fuc*p*-(1→3)]-D-GlcNAc, and α-NeuAc-(2→6)-β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc-(1→3)-β-D-Gal*p*-(1→4)-[α-L-Fuc*p*-(1→3)]-β-D-GlcNAc.

INTRODUCTION

The biology of UDP-D-galactose:β-*N*-acetylglucosaminyl (1→4)-β-D-galactosyltransferase (EC 2.4.1.22) has recently been reviewed[1]. Despite the fact that methods of purification of this enzyme were available fifteen years ago[2], its structure remains unknown. The soluble, human-milk galactosyltransferase, which is a glycoprotein of M_r 55 000, has been purified to apparent homogeneity as judged by $NaDodSO_4$–PAGE[3]. However, the enzyme was resolved into at least thirteen bands by isoelectric focusing. All these bands corresponded to enzymically active

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protein isoforms reflecting extensive microheterogeneity of galactosyltransferase. Possibly, this heterogeneity prevented sequence analysis. Furthermore, there is little information on the structures of the carbohydrate moiety of galactosyltransferase except for the enzyme of bovine milk[4] and human serum[5]. We report herein the detailed carbohydrate structure of galactosyltransferase isolated from human milk as part of a complete structural analysis of this enzyme. Main structures of asparagine-linked oligosaccharides are proposed and evidence for extensive microheterogeneity in the carbohydrate moiety is given.

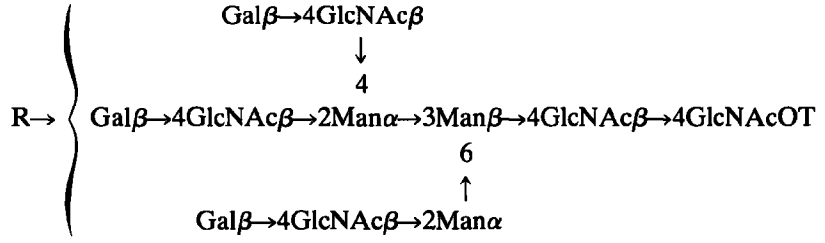
EXPERIMENTAL

Materials. — Human milk from a single donor, D. T. of blood-group O^+, nonsecretor, Le^a, was collected over a period of several months during lactation and stored at −20°. Galactosyltransferase was isolated as previously described by acid precipitation of casein at pH 4.6, and two sequential affinity chromatographies on GlcNAc– and human α-lactalbumin–Sepharose columns. The isolated enzyme was pooled, dialyzed against water, and lyophilized. NaB^3H_4 (12.6 GBq/mmol) was purchased from New England Nuclear, Boston, MA, and NaB^2H_4 and sialidase from *Arthrobacter ureafaciens*[6] were purchased from Nakarai Chemicals, Ltd., Kyoto. β-D-Galactosidase, *N*-acetyl-β-D-hexosaminidase, and α-D-mannosidase were purified from jack bean meal by the method of Li and Li[7]. The α-D-mannosidase, at a concentration lower than 8 munits/μL, released one mannose residue from R→Manα→6(Manα→3)Manβ→4GlcNAcβ→4GlcNAcOT* but not from Manα→6(R→Manα→3)Manβ→4GlcNAcβ→4GlcNAcOT in which R represents sugar groups[8]. At higher enzyme concentration, it cleaved Manα→6(R→Manα→3)Manβ→4GlcNAcβ→4GlcNAcOT as well. Diplococcal β-D-galactosidase and *N*-acetyl-β-D-hexosaminidase were purified from the culture fluid of *Diplococcus pneumoniae* according to the method of Glasgow *et al.*[9]. The diplococcal β-D-galactosidase cleaved the β-D-Gal*p*-(1→4)-D-GlcNAc linkage but could not cleave the β-D-Gal*p*-(1→3)-D-GlcNAc and β-D-Gal*p*-(1→6)-D-GlcNAc linkages[10]. The diplococcal *N*-acetyl-β-D-hexosaminidase could not cleave the β-D-Glc*p*NAc-(1→4)-D-Man and β-D-Glc*p*NAc-(1→6)-D-Man linkages, although it cleaved the β-D-Glc*p*NAc-(1→2)-D-Man linkage. The β-D-Glc*p*NAc-(1→2)-D-Man linkage of the β-D-Glc*p*NAc-(1→4)-[β-D-Glc*p*NAc-(1→2)]-D-Man group was cleaved by the enzyme, but that of the β-D-Glc*p*NAc-(1→6)-[β-D-Glc*p*NAc-(1→2)]-D-Man group was not[11]. In contrast, jack bean β-D-galactosidase and jack bean *N*-acetyl-β-D-hexosaminidase could cleave all β-D-galactopyranosyl and 2-acetamido-2-deoxy-β-D-glucopyranosyl linkages under the condition used in this study. Three α-L-fucosidases having different substrate specificities were used in

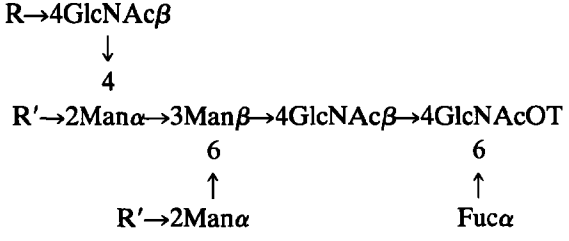
*In abbreviated formulas, the pyranose form, glycosyl linkages at C-1 (C-2 for NeuAc), and D-configuration (L-configuration for fucose) are assumed; GlcNAcOT corresponds to 2-acetamido-2-deoxy-D-[1-^{3}H]glucitol.

this study; bovine epididymis α-L-fucosidase, which cleaves all α-L-fucopyranosyl linkages was purchased from Sigma, St. Louis, MO; *Bacillus fulminans* α-fucosidase, which cleaves only the Fucα→2Gal linkage, was purified according to the method of Kochibe[12]; and α-fucosidase I, which cleaves the α-L-fucopyranosyl linkages of the β-D-Gal*p*-(1→4)-[α-L-Fuc*p*-(1→3)]-D-GlcNAc and β-D-Gal*p*-(1→3)-[α-L-Fuc*p*-(1→4)]-D-GlcNAc but not of the α-L-Fuc*p*-(1→2)-D-Gal and the α-L-Fuc*p*-(1→6)-D-GlcNAc groups[13], was purified from almond emulsin as reported previously[14]. Snail β-D-mannosidase was kindly supplied by Seikagaku Kogyo Co., Tokyo. One unit of glycosidases was defined as the amount of enzyme required to hydrolyze 1 μmol of *p*-nitrophenyl glycosides or ^{3}H-labeled standard oligosaccharides per min. D-Glucose oligomers were prepared by partial acid hydrolysis of dextran[15].

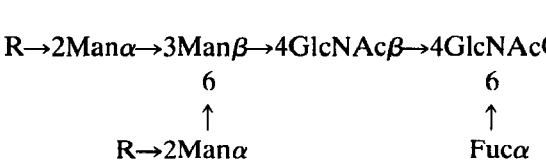
Standard oligosaccharides. — The following oligosaccharides were obtained as follows: The monosialyl derivative of **1** (**2**) from human ceruloplasmin[16]; **3, 6,** and **9** from hamster melanoma tyrosinase[17]; **10** and **11** from human parotid α-amylase[8]; **4** from **3** by incubation with a mixture of diplococcal β-D-galactosidase and diplococcal *N*-acetyl-β-D-hexosaminidase; **7** and **8** by sequential digestion of **6** with jack bean β-D-galactosidase and jack bean *N*-acetyl-β-D-hexosaminidase, respectively; and **5** from **3** by jack bean β-galactosidase digestion.



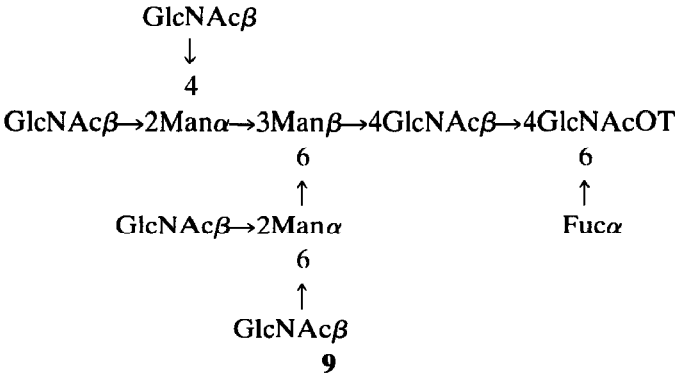
1 R = H
2 R = NeuAcα_1



3 R = Galβ, R' = Galβ→4GlcNAcβ
4 R = H, R' = H
5 R = H, R' = GlcNAcβ



6 R = Galβ→4GlcNAcβ
7 R = GlcNAcβ
8 R = H



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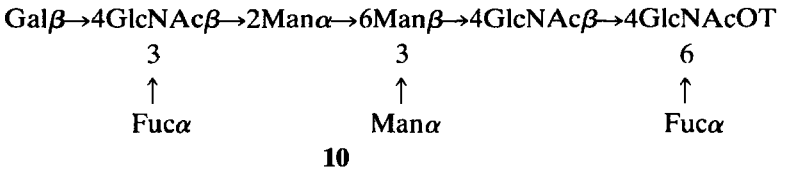

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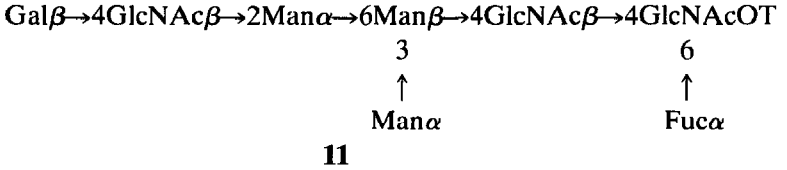

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Glycosidase digestion of ^{3}H-labeled oligosaccharides. — Unless otherwise noted, ^{3}H-labeled oligosaccharides (1–10 × 10^4 c.p.m.) were incubated with one of the following mixtures at 37° for 18 h: (a) jack bean β-D-galactosidase digestion, enzyme (0.5 unit) in 50mM sodium citrate buffer (pH 3.5, 40 μL); (b) jack bean *N*-acetyl-β-D-hexosaminidase digestion, enzyme (0.5 unit) in 50mM sodium acetate buffer (pH 4.5, 50 μL); (c) diplococcal β-D-galactosidase digestion, enzyme (20 munits) in 0.1M citrate–phosphate buffer (pH (6.0, 50 μL); (d) diplococcal *N*-acetyl-β-D-hexosaminidase digestion, enzyme (4 munits) in 0.1M citrate–phosphate buffer (pH 6.0, 50 μL); (e) jack bean β-D-galactosidase and jack bean *N*-acetyl-β-D-hexosaminidase digestion, a mixture of β-D-galactosidase (0.5 unit) and *N*-acetyl-β-

D-hexosaminidase (0.5 unit) in 0.1M citrate–phosphate buffer (pH 4.0, 50 μL); (f) sialidase digestion, enzyme (0.1 unit) in 0.1M acetate buffer (pH 5.0, 50 μL); (g) almond α-L-fucosidase I digestion, enzyme (40 μunits) in 0.15M citrate–phosphate buffer (pH 5.0, 30 μL); (h) *Bacillus* α-L-fucosidase digestion, enzyme (17.5 μg) in 50mM Na_2HPO_4 buffer (pH 6.6, 30 μL); and (i) α-D-mannosidase digestion, enzyme (0.5 unit) in 0.1M acetate buffer (pH 4.5, 60 μL). One drop of toluene was added to all reaction mixtures to inhibit bacterial growth. Reactions were terminated by heating the reaction mixture in a boiling water for 2 min and the products were analyzed by Bio-Gel P-4 column chromatography.

Analytical methods. — Radioactivity was determined with an Aloka liquid-scintillation spectrometer model LSC-700. Radiochromatoscanning was performed with Packard radiochromatoscanner model 7201. Methylation analysis of oligosaccharides was performed as described in the previous paper[18]. Identification of sialic acid released from galactosyltransferase was performed by paper chromatography using 12:3:5 butanol–acetic acid–water after NaB^3H_4 reduction[19]. High-voltage paper electrophoresis was performed in pyridine–acetate buffer, pH 5.4 (3:1:387 pyridine–acetic acid–water) at 73 V/cm for 1.5 h. Fractionation of radioactive oligosaccharides by Bio-Gel P-4 column chromatography was performed as reported by Yamashita *et al.*[20].

Liberation of the asparagine-linked sugar chains from human milk galactosyltransferase. — Thoroughly dried galactosyltransferase (10 mg) was subjected to hydrazinolysis for 10 h as previously described[21]. An aliquot ($^1/_5$) of the oligosaccharide fraction was reduced with NaB^3H_4 (1.2 μmol, 14.8 MBq) in 50mM NaOH (100 μL) at 30° for 4 h, and the resulting radioactive oligosaccharide was purified as already described[21]. For methylation analysis, the remaining oligosaccharide was reduced with NaB^2H_4 in the same manner as in the case of NaB^3H_4 reduction. To facilitate detection of the oligosaccharides, half of the tritium-labeled oligosaccharide fraction was added to the deuterium-labeled sample.

The yield of the total radioactive oligosaccharides from 1.0 mg of the galactosyltransferase was 4.5×10^5 c.p.m. On the basis of the specific activity of NaB^3H_4 used and the mol wt. of 55 000 of the galactosyltransferase, it was concluded that the enzyme contains one asparagine-linked sugar chain per molecule.

RESULTS

Fractionation of oligosaccharides by paper electrophoresis. — The radioactive oligosaccharides mixture obtained from human milk galactosyltransferase by hydrazinolysis was separated into a neutral (**N**) and an acidic (**A**) fraction by paper electrophoresis (Fig. 1-I). When the acidic fraction was incubated with sialidase, it was completely converted into a neutral oligosaccharides mixture, indicating that only sialic acids are included as the acidic residues (data not shown). This desialylated oligosaccharides mixture (**AN**) was recovered from the paper electrophoretogram. The sialic acid residues released from the intact galactosyltransferase

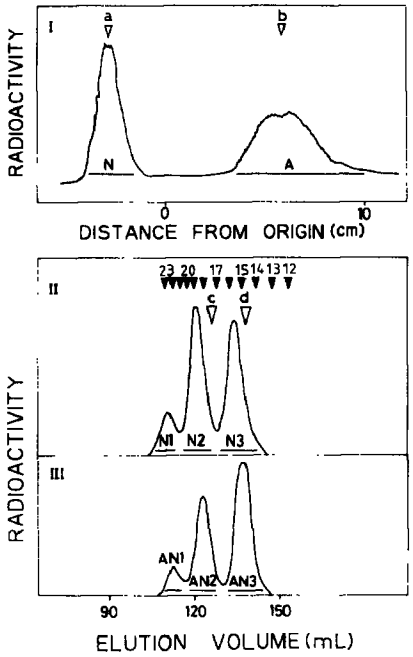


Fig. 1. Fractionation of radioactively labelled oligosaccharides liberated from human milk galactosyltransferase by hydrazinolysis. The oligosaccharide fraction was subjected to paper electrophoresis at pH 5.4 (I). The neutral fraction **N** and the fraction **AN** obtained by sialidase digestion of fraction **A** were subjected to Bio-Gel P-4 column chromatography and the elution profiles are shown in panels II and III, respectively. In panel I, the white triangles indicate the elution positions of standard oligosaccharides: (a) lactitol, and (b) **2**. In panel II, the black triangles indicate the elution positions of glucose oligomers and the numerals the numbers of glucose units. The white triangles indicate the elution positions of authentic radioactively labeled oligosaccharides, (c) **3** and (d) **6**.

by sialidase digestion were all *N*-acetylneuraminic acid and no *N*-glycolylneuraminic acid was detected.

The mobility of the acidic peak was the same as that of authentic **2** indicating that the acidic oligosaccharides were all monosialylated. The observation that no additional acidic radioactive component was produced by partial desialylation also supported this conclusion (data not shown). However, the acid peak was expected to be a mixture of various oligosaccharides with different sizes because it was very broad in the electrophoretogram. This was confirmed by the structural studies discussed later. The molar ratio of oligosaccharides in fractions **N** and **A**, calculated on the basis of their radioactivities, was 11:14.

Fractionation of neutral oligosaccharides by Bio-Gel P-4 column chromatography. — Fractions **N** and **AN** were subjected to Bio-Gel P-4 column chromatography. As shown in Fig. 1-II, fraction **N** was separated into three radioactive components (**N1, N2**, and **N3**) with elution positions of 22.5, 19.0, and 15.5 glucose units, respectively, and a molar ratio, calculated on the basis of the radioactivity, of 12:43:45. Fraction **AN** was also separated into three components (**AN1, AN2**,

TABLE I

METHYLATION ANALYSIS OF OLIGOSACCHARIDES OF FRACTIONS **A, AN,** AND **N**

Methylated sugar	*Fraction (molar ratio)*[a]		
	A	**AN**	**N**
Fucitol			
2,3,4-Tri-*O*-methyl-(1,5-di-*O*-acetyl)	2.7	2.6	3.5
Galactitol			
2,3,4,6-Tetra-*O*-methyl-(1,5-di-*O*-acetyl)	1.3	2.4	2.4
2,3,4-Tri-*O*-methyl-(1,5,6-tri-*O*-acetyl)	1.0		
2,4,6-Tri-*O*-methyl-(1,3,5-tri-*O*-acetyl)	0.3	0.3	0.3
Mannitol			
3,4,6-Tri-*O*-methyl-(1,2,5-tri-*O*-acetyl)	1.4	1.2	1.5
3,6-Di-*O*-methyl-(1,2,4,5-tetra-*O*-acetyl)	0.5	0.5	0.5
2,4-Di-*O*-methyl-(1,3,5,6-tetra-*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	0.7	0.8
3,6-Di-*O*-methyl-(1,4,5-tri-*O*-acetyl)	1.6	1.6	0.7
6-Mono-*O*-methyl-(1,3,4,5-tetra-*O*-acetyl)	1.2	1.2	2.6

[a]Relative to the value of 2,4-di-*O*-methylmannitol taken as 1.0.

and **AN3**) with elution positions of 22.0, 18.7, and 15.2 glucose units, respectively, and a molar ratio of 12:35:53 (Fig. 1-III).

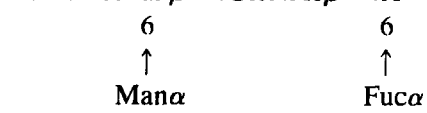
Methylation analysis of the oligosaccharides fractions. — As will be described later, human milk galactosyltransferase contains quite a variety of sugar chains. Since it was almost impossible to obtain methylation data for individual oligosaccharides, the elucidation of the structure of each oligosaccharide was based mostly on sequential exoglycosidase digestion. However, for interpretation of the results, a knowledge of the sugar linkages present in the oligosaccharides was required and, thus, fractions **N, AN,** and **A** were subjected to methylation analysis.

Neither 2-mono-*O*-methyl- nor 2,3,4,6-tetra-*O*-methyl-mannitol was found in the three samples. These results indicated that the oligosaccharides in the three fractions are all of the complex-type chains without a 2-acetamido-2-deoxy-D-glucopyranosyl group linked to O-4 of the β-D-mannopyranosyl residue ("bisecting group"). This was confirmed by sequential exoglycosidase digestion as will be described later. Therefore, the numbers of partially *O*-methylated monosaccharide obtained from the three samples were calculated by taking the values of 2,4-di-*O*-methylmannitol as 1.0 (see Table I).

Comparison of the data for fractions **A** and **AN** indicated that all *N*-acetylneuraminic acid groups in the acidic oligosaccharides are linked at O-6 of the subterminal galactose units. Detection of 2,3,4,6-tetra-*O*-methylgalactitol in fraction **A** indicated that a substantial proportion of the galactose units of the acidic oligosaccharides are not sialylated. Since fucitol was detected only as the 2,3,4-tri-*O*-methyl derivative in all three samples, the L-fucosyl groups in the sugar chains of human milk galactosyltransferase all occur as nonreducing groups. All oligo-

saccharides have an 2-acetamido-2-deoxy-D-glucose residue at the reducing end and this residue is substituted at O-6 probably by a fucosyl group, because 2-deoxy-1,3,5-tri-*O*-methyl- but no 2-deoxy-1,3,5,6-tetra-*O*-methyl-2-(*N*-methylacetamido)-deoxyglucitol was found in all three samples.

The data of fractions **N** and **AN** indicated that the core portions of the oligosaccharides in these fractions had structure **12** and that the two α-D-mannopyranosyl residues are mostly substituted either at O-2 or O-2,4 by outer chains. Detection of 2-deoxy-3,6-di- and 2-deoxy-6-*O*-methyl-2-(*N*-methylacetamido)-glucitol indicated that the internal 2-acetamido-2-deoxy-D-glucose residues occur either linked at O-4, or O-3 and -4. Detection of 2,4,6-tri-*O*-methylgalactitol in the two neutral fractions indicated that some of the outer chain moieties of the oligosaccharides may contain β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc-(1→3) repeating structures. The results so far described for the methylation study indicated that no unusual sugar linkage is present in the oligosaccharides released from human milk galactosyltransferase, and the previously described substrate-specific exoglycosidases[8,10–13] may be used for the structural studies of these oligosaccharides.



12 X = GlcNAc
13 X = GlcNAcOT

Structural studies of oligosaccharides of fraction **N1** *and of the major oligosaccharides of fractions* **N2** *and* **N3**. — When fraction **N3** was incubated with jack bean β-D-galactosidase, one D-galactosyl group was removed from 18% of the fraction and the remaining part was completely resistant to enzyme digestion (data not shown). When this incubation mixture was then digested with jack bean *N*-acetyl-β-D-hexosaminidase, one 2-acetamido-2-deoxy-D-glucosyl group was released from the degalactosylated product, while the portion of fraction **N3** resistant to the β-D-galactosidase treatment was also resistant to this enzymic digestion (Fig. 2A). These results indicated that fraction **N3** contains a mixture of at least two different oligosaccharides. The oligosaccharides that were resistant and susceptible to β-D-galactosidase were named **N3-1** and **N3-2**, respectively.

Fraction **N3-1** (peak I in Fig. 2A) was not degraded by *Bacillus* α-L-fucosidase (data not shown). However, almond α-L-fucosidase I released two fucosyl groups to give a radioactive oligosaccharide having the same mobility as authentic **6** (Fig. 2B). The radioactive product illustrated in Fig. 2B released two D-galactosyl groups on incubation with diplococcal β-D-galactosidase (Fig. 2C), and two 2-acetamido-2-deoxy-D-glucosyl groups on subsequent diplococcal *N*-acetyl-β-D-hexosaminidase digestion (Fig. 2D). The mobility of the radioactive peak

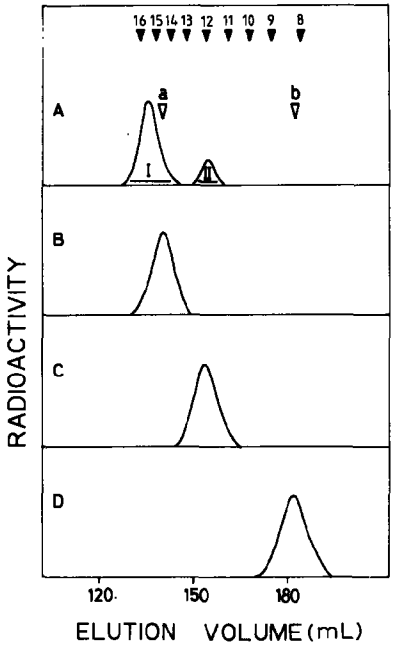
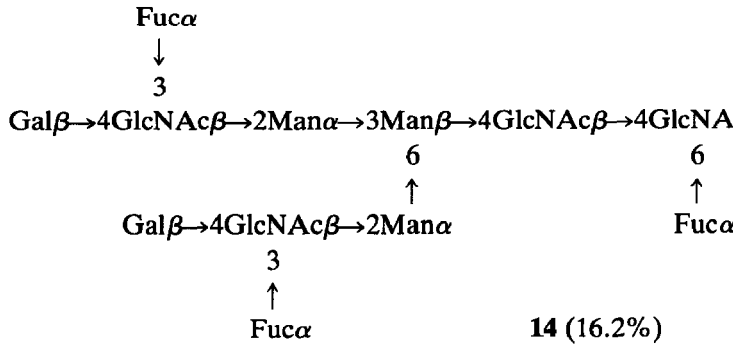


Fig. 2. Sequential exoglycosidase digestion of radioactively labeled fraction **N3**. The radioactively labeled sugars were analyzed by Bio-Gel P-4 column chromatography. Analytical conditions and black triangles are the same as in the legend to Fig. 1-II. The white triangles indicate the elution positions of standard oligosaccharides: (a) **6** and (b) **8**. (A) Radioactively labeled fraction **N3** incubated with jack bean β-D-galactosidase and then with jack bean *N*-acetyl-β-D-hexosaminidase; (B) oligosaccharide **14** (the peak I in panel A) incubated with almond α-L-fucosidase I; (C) the radioactively labeled peak in panel B incubated with diplococcal β-D-galactosidase; and (D) the radioactively labeled peak in panel C incubated with diplococcal *N*-acetyl-β-D-hexosaminidase.

illustrated in Fig. 2D was the same as authentic **8**. Structure **13** for the radioactively labeled hexaglycitol was confirmed by sequential digestion with α-D-mannosidase, β-D-mannosidase, jack bean *N*-acetyl-β-D-hexosaminidase, and epididymal α-L-fucosidase (data not shown). In fraction **N3-1**, two outer chains composed of one D-galactose, one 2-acetamido-2-deoxy-D-glucose, and one L-fucose units are linked to this hexaglycitol core. The structure of the trisaccharide group is β-D-Gal*p*-(1→4)-[α-L-Fuc*p*-(1→3)]-β-D-GlcNAc, as diplococcal β-D-galactosidase cleaves only the β-D-Gal*p*-(1→4)-D-GlcNAc linkage[10] and almond α-L-fucosidase I the α-L-fucosyl linkages of the β-D-Gal*p*-(1→4)-[α-L-Fuc*p*-(1→3)]-D-GlcNAc and β-D-Gal*p*-(1→3)-[α-L-Fuc*p*-(1→4)]-D-GlcNAc groups[13]. These trisaccharide outer chains are linked at O-2 of the two α-D-mannopyranosyl residues of the core hexaglycitol, as diplococcal *N*-acetyl-β-D-hexosaminidase cleaves only the β-D-Glc*p*NAc-(1→2)-D-Man linkage[11]. Based on the data so far described, structure **14** is proposed for the oligosaccharide of fraction **N3-1**.



14 (16.2%)

When radioactively labeled fraction **N2** was incubated with jack bean β-D-galactosidase, 12% of the fraction was converted into a lower mol. wt. oligosaccharide having the mobility of 17.9 glucose units by the release of one galactosyl group, and the remaining 88% of the fraction remained intact (data not shown). When this incubation mixture was then digested with jack bean *N*-acetyl-β-D-hexosaminidase, the degalactosylated oligosaccharide was converted into a radioactively labeled oligosaccharide having the mobility of 15.7 glucose units by the release of

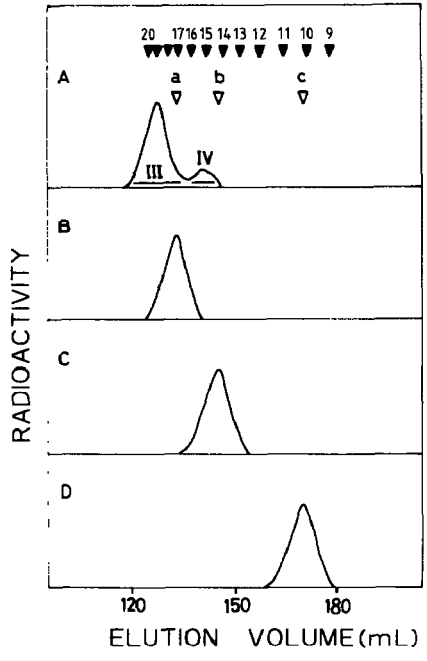
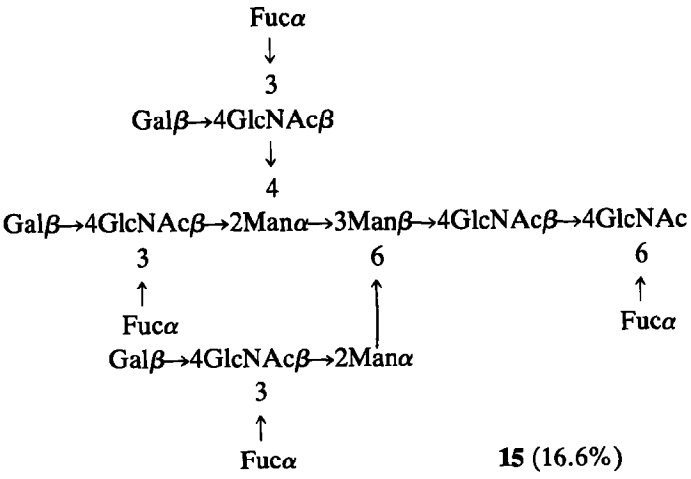


Fig. 3. Sequential exoglycosidase digestion of radioactively labeled fraction **N2**. Analytical conditions and black triangles are the same as in the legend to Fig. 1-II. The white triangles indicate the elution positions of standard oligosaccharides: (a) **3**, (b) **5**, and (c) **4**. (A) Radioactively labeled fraction **N2** incubated with jack bean β-D-galactosidase and then with jack bean *N*-acetyl-β-D-hexosaminidase; (B) oligosaccharide **15** (the peak III in panel A) incubated with almond α-L-fucosidase I; (C) the radioactively labeled peak in panel B incubated with diplococcal β-D-galactosidase; and (D) the radioactively labeled peak in panel C incubated with diplococcal *N*-acetyl-β-D-hexosaminidase.

one 2-acetamido-2-deoxy-D-glucopyranosyl group, and the oligosaccharide fraction resistant to β-D-galactosidase treatment was also resistant to *N*-acetyl-β-D-hexosaminidase (Fig. 3A). The oligosaccharides in fraction **N2** resistant and susceptible to the enzymic digestion were named **N2-1** and **N2-2**, respectively.

Fraction **N2-1** was resistant to *Bacillus* α-L-fucosidase (data not shown), but it was converted into a radioactively labeled oligosaccharide having the same mobility as authentic **3** by incubation with almond α-L-fucosidase I (Fig. 3B). The difference in mobilities of the radioactive peaks in Fig. 3A and 3B was approximately 2.1 glucose units. Since the effective size of the α-L-fucosyl groups, relative to glucose, in β-D-Gal*p*-(1→4)-[α-L-Fuc*p*-(1→3)]-D-GlcNAc and β-D-Gal*p*-(1→3)-[α-LFuc*p*-(1→4)]-D-GlcNAc groups is 0.7, approximately three fucosyl groups were released by the α-L-fucosidase digestion. The radioactively labeled oligosaccharide illustrated in Fig. 3B was converted into an oligosaccharide having the same mobility as authentic **5** by incubation with diplococcal β-D-galactosidase (Fig. 3C), which was then converted into **8** by incubation with jack bean *N*-acetyl-β-D-hexosaminidase (data not shown). When the radioactively labeled oligosaccharide illustrated in Fig. 3C was incubated with diplococcal *N*-acetyl-β-D-hexosaminidase, only two 2-acetamido-2-deoxy-D-glucosyl groups were released (Fig. 3D). The radioactively labeled oligosaccharide illustrated in Fig. 3D was completely resistant to the second diplococcal *N*-acetyl-β-D-hexosaminidase treatment (data not shown). These results indicated that the three outer-chain 2-acetamido-2-deoxy-D-glucosyl groups of the oligosaccharide illustrated in Fig. 3C occur as β-D-Glc*p*NAc-(1→2)-α-D-Man and β-D-Glc*p*NAc-(1→4)-[β-D-Glc*p*NAc-(1→2)]-α-D-Man groups.

When the radioactively labeled oligosaccharide illustrated in Fig. 3D was in-



15 (16.6%)

cubated with α-D-mannosidase, no mannose residue was removed (data not shown). This result indicated that the β-D-Glc*p*NAc-(1→4)-[β-D-Glc*p*NAc-(1→2)]-D-Man group is exclusively located on the α-D-Man-(1→3) chain of the trimannosyl core. These results together with the methylation data described earlier indicated that the oligosaccharide of fraction **N2-1** has structure **15**.

When fraction **N1** was incubated with almond α-L-fucosidase I, it was converted into a product giving a broad radioactive peak that was eluted at 20.5 glucose units (Fig. 4A). This result indicated that approximately three to four L-fucosyl groups had been removed by the enzymic digestion. Incubation of this product with diplococcal β-D-galactosidase resulted in a pair of a major and a minor radioactive peaks (Fig. 4B). The mobility of the major peak (Peak V) was 18.5 glucose units, indicating that at least two galactosyl groups had been removed. The minor peak (Peak VI) migrated at the same position as authentic **9**, indicating that

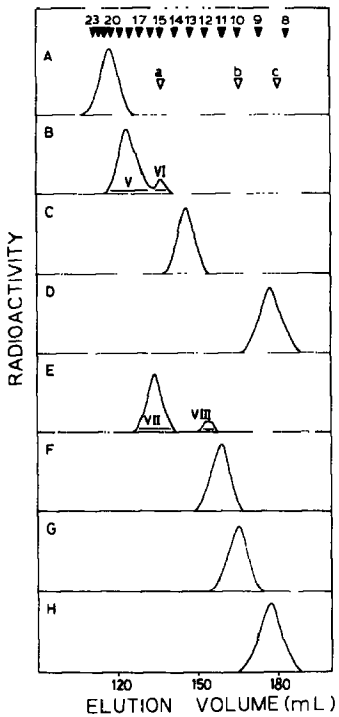
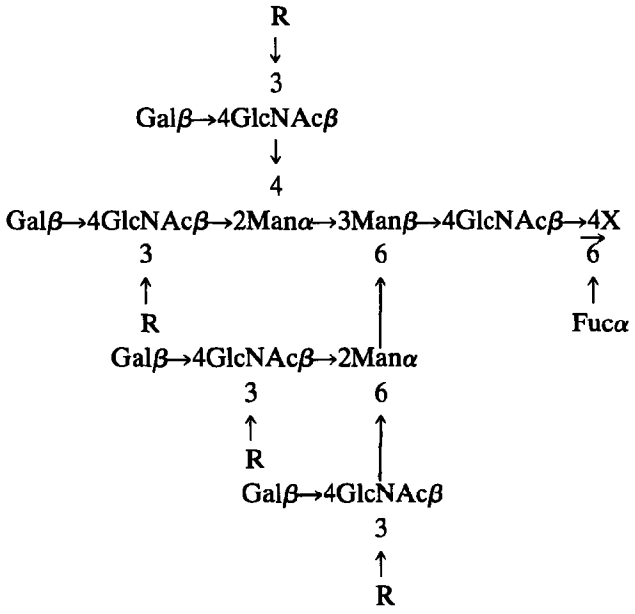


Fig. 4. Sequential exoglycosidase digestion of radioactive labeled fraction **N1**. Analytical conditions and black triangles are the same as in the legend to Fig. 1-II. The white triangles (a, b, and c) indicate the elution positions of authentic **9**, **4**, and **8**, respectively. (A) Radioactively labeled fraction **N1** incubated with almond α-L-fucosidase I; (B) the radioactive labeled peak in panel A incubated with diplococcal β-D-galactosidase; (C) the radioactively labeled peak VI in panel B incubated with diplococcal *N*-acetyl-β-D-hexosaminidase; (D) the radioactively labeled peak in panel C incubated with jack bean *N*-acetyl-β-D-hexosaminidase; (E) the radioactively labeled peak V in panel (B) incubated with diplococcal *N*-acetyl-β-D-hexosaminidase; (F) the radioactively labeled peak VIII in panel E incubated with almond α-L-fucosidase I; (G) the radioactively labeled peak in panel F incubated with diplococcal β-D-galactosidase; and (H) the radioactively-labeled peak in panel G incubated with jack bean *N*-acetyl-β-D-hexosaminidase.

four D-galactosyl group had been removed. This minor product (Fig. 4B) released one 2-acetamido-2-deoxy-D-glucosyl group on incubation with diplococcal *N*-acetyl-β-D-hexosaminidase (Fig. 4C) and three additional 2-acetamido-2-deoxy-D-glucosyl groups on treatment with jack bean *N*-acetyl-β-D-hexosaminidase (Fig. 4D). The elution position of the radioactive peak in Fig. 4D was the same as that of authentic **8**. These results indicated that the minor peak of Fig. 4B was derived from the tetraantennary oligosaccharide **16**. A few additional comments are necessary for the occurrence of the tetraantennary oligosaccharide. As shown in Table I, no 3,4-di-*O*-methylmannitol was detected in the methylation study. However, the molar ratio of the minor peak of Fig. 4B to the total oligosaccharides is only 0.5%. Therefore, the amount of di-*O*-methylmannitol might be below the limit of detection. In fraction **N1**, this tetraantennary oligosaccharide may occur as a tri- or tetra-fucosylated compound (**17**).

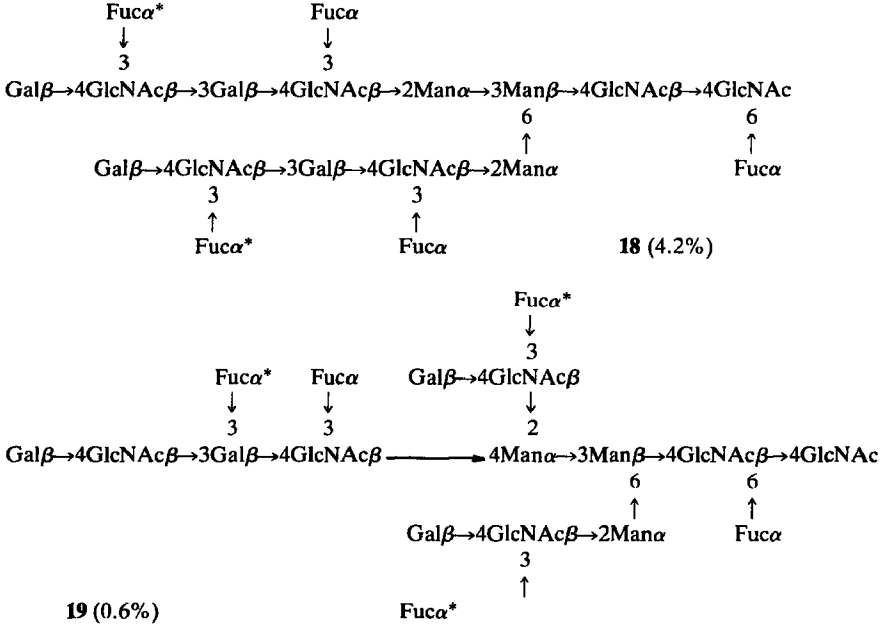


16 X = GlcNAcOT, R = H
17 X = GlcNAc, R = Fucα* (0.5%)

When oligosaccharides of peak V (Fig. 4B) were incubated with diplococcal *N*-acetyl-β-D-hexosaminidase, they were again converted into a pair of major (15.5 glucose units) and minor component (11.7 glucose units) by the release of two and three 2-acetamido-2-deoxy-D-glucosyl groups, respectively (Fig. 4E). No additional group was released from the major and the minor components by further treatment

*Some of these L-fucopyranosyl groups marked by an asterisk may be absent in these structures.

with jack bean *N*-acetyl-β-D-hexosaminidase (data not shown). The product of the major peak (Peak VII, Fig. 4E) gave exactly the same sequential exoglycosidase digestion pattern as that of fraction **N3-1** (Peak I, Fig, 2A) described earlier. Therefore, the two outer chains of the major component are considered to occur in fraction **N1** as Galβ→4(±Fucα→3)GlcNAcβ→3Galβ→4(Fucα→3)GlcNAcβ→2 as shown by structure **18**. The minor component (Peak VIII, Fig. 4E) released one



L-fucosyl group on incubation with almond α-L-fucosidase I (Fig. 4F), but was totally resistant to α-D-mannosidase digestion (data not shown). When this radioactively labeled product (Fig. 4F) was incubated with diplococcal β-D-galactosidase, it was converted into an oligosaccharide having the same mobility as authentic **4** by the release of one D-galactosyl group (Fig. 4G). Not diplococcal but jack bean *N*-acetyl-β-D-hexosaminidase digestion converted this product (Fig. 4G) into **8** (Fig. 4H), indicating that the minor component originally occur in fraction **N1** as a triantennary oligosaccharide having two Galβ→4(±Fucα→3)GlcNAc and one Galβ→4(±Fucα→3)GlcNAcβ→3Galβ→4(Fucα→3)GlcNAc groups in its outer chains (see structure **19**). The results of diplococcal and jack bean *N*-acetyl-β-D-hexosaminidase digestion indicated that the β-D-Gal*p*-(1→4)-D-GlcNAc repeating unit in the outer chain was exclusively located at O-4 of an α-D-mannosyl residue (see structure **19**). Since approximately 15% of the original fraction **N1** released one D-galactosyl and one 2-acetamido-2-deoxy-D-glucosyl group on incubation with a mixture of jack bean β-D-galactosidase and jack bean *N*-acetyl-β-D-hexosaminidase (data not shown), one terminal group β-D-Gal*p*-(1→4)-D-GlcNAc of 15% of the oligosaccharides in fraction **N1** is not fucosylated.

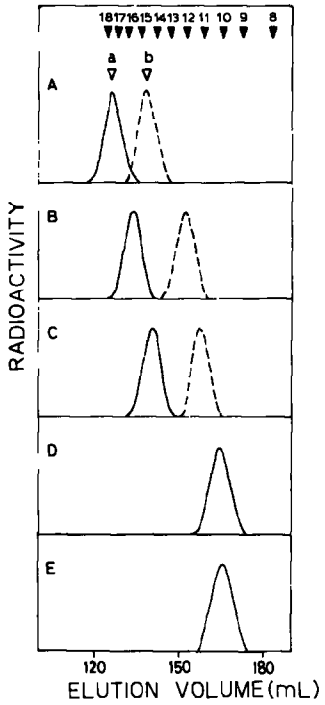
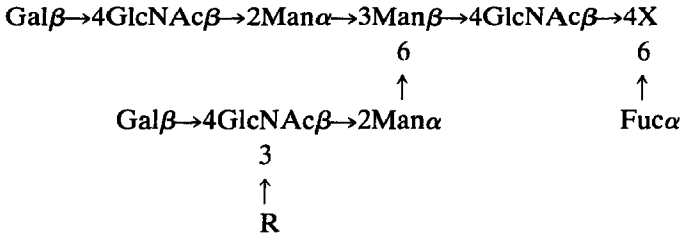


Fig. 5. Sequential exoglycosidase digestion of radioactively labeled fractions **AN3** (-----) and **AN2**(——). Analytical conditions and black triangles are the same as in the legend to Fig. 1-II. White triangles indicate the elution positions of authentic oligosaccharides: (a) **3** and (b) **6**. (A) Oligosaccharides fractions **AN3** (**21**) and **AN2** (**22**) were incubated with almond α-L-fucosidase I; (B) oligosaccharides fractions **AN3** and **AN2** incubated with a mixture of jack bean β-D-galactosidase and jack bean *N*-acetyl-β-D-hexosaminidase; (C) the radioactively labeled peaks in panel B incubated with almond α-L-fucosidase I; (D) the peak indicated by the dotted line peak in panel C incubated with α-D-mannosidase; and (E) the peak indicated by the solid line peak in panel C incubated with a mixture of diplococcal β-D-galactosidase and diplococcal *N*-acetyl-β-D-hexosaminidase.

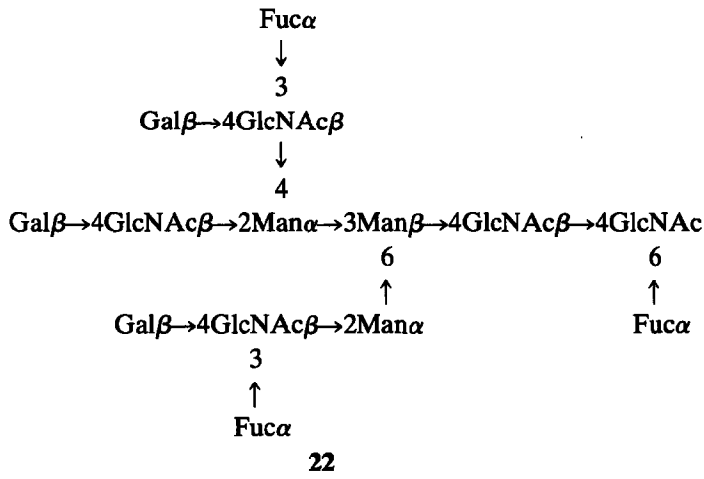
Structures of oligosaccharides in fractions **AN2** *and* **AN3**. — Incubation of radioactively labeled fraction **AN3** with almond α-L-fucosidase I released one fucosyl group to give a radioactively labeled oligosaccharide having the same mobility as authentic **6** (dotted line, Fig. 5A). Further sequential exoglycosidase digestion gave exactly the same results as that illustrated in Fig. 2B, suggesting structure **20** for the radioactively labeled oligosaccharide. Incubation of fraction **AN3** with a mixture of jack bean β-D-galactosidase and jack bean *N*-acetyl-β-D-hexosaminidase released one D-galactosyl and one 2-acetamido-2-deoxy-D-glucosyl group to give a radioactively labeled oligosaccharide having the mobility of 12.0 glucose units (dotted line, Fig. 5B). When this product was treated with almond α-L-fucosidase I, one L-fucosyl group was released (dotted line, Fig. 5C), and one D-mannosyl group was released by further incubation with α-D-mannosidase (Fig. 5D). These results indicated that the L-fucosyl group in the outer chain of the oligosaccharide of fraction **AN3** is linked to the 2-acetamido-2-deoxy-D-glucosyl

residue of the β-D-Gal*p*-(1→4)-D-GlcNAc group which is part of the chain linked (1→6) to the β-D-mannosyl residue and suggest structure **21**.



20 X = GlcNAcOT, R = H
21 X = GlcNAc, R = Fucα

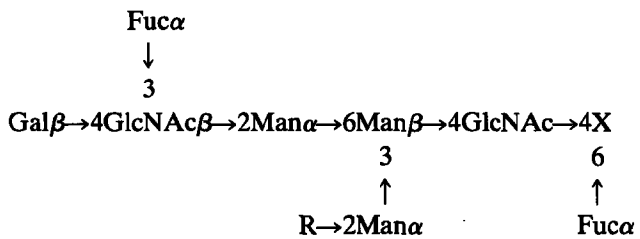
Two L-fucosyl groups were released from radioactively labeled fraction **AN2** by incubation with almond α-L-fucosidase I (solid line in Fig. 5A). The mobility of the radioactive product was the same as that of authentic **3**. This radioactive product gave exactly the same results of sequential exoglycosidase digestion as the radioactive component illustrated in Fig. 3B. Therefore, fraction **AN2** was considered to contain a triantennary oligosaccharide having two Galβ→4(Fucα→3)GlcNAc outer chains and one Galβ→4GlcNAc outer chain. In order to assign the location of the nonfucosylated outer chain, radioactively labeled fraction **AN2** was incubated with a mixture of jack bean β-D-galactosidase and jack bean *N*-acetyl-β-D-hexosaminidase. The enzymic digestion product gave a single radioactive peak at 15.5 glucose units (solid line in Fig. 5B), indicating that a Galβ→4GlcNAc group had been removed. The two α-L-fucosyl groups in the outer chain were then removed by almond α-L-fucosidase I digestion (solid line in Fig. 5C). The resulting radioactively labeled decasaccharide was completely resistant to a high concentration (40 munits/μL) of α-D-mannosidase (data not shown), indicating that the two Galβ→4GlcNAc groups are linked to two α-D-mannosyl residues of the core. Sequential digestion with diplococcal β-D-galactosidase and diplococcal *N*-acetyl-β-D-hexosaminidase removed two D-galactose and one 2-acetamido-2-deoxy-D-glucose residues from the decasaccharide (Fig. 5E). These results indicated that one Galβ→4GlcNAc group of this decasaccharide is linked at O-2 and the other Galβ→4GlcNAc group at O-4 of the α-D-mannosyl residues. The radioactively labeled oligosaccharide illustrated in Fig. 5E was still resistant to α-D-mannosidase digestion (data not shown). This result indicated that the Galβ→4(Fucα→3)GlcNAcβ→4 group is exclusively located on the Manα→3 chain. Accordingly, the Galβ→4(Fucα→3)GlcNAcβ→2 group is linked to the Manα→6 residue of the trimannosyl core, suggesting structure **22** for the oligosaccharide of fraction **AN2**.



22

Structural studies of minor oligosaccharides of fractions **N2, N3,** *and* **AN1.** — Because of the extremely small amount of the samples, the structures of the oligosaccharides described in this section are not completely conclusive.

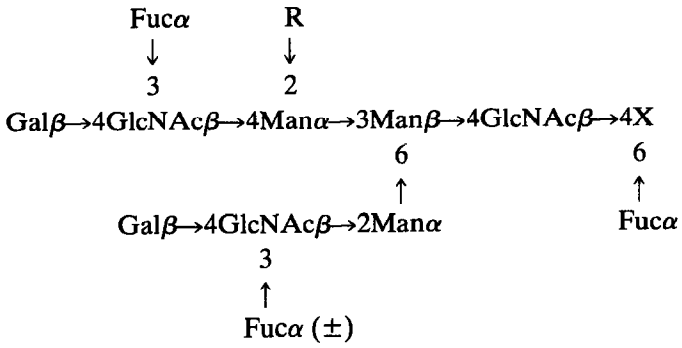
The product obtained by sequential digestion of fraction **N3-2** with jack bean β-D-galactosidase and jack bean *N*-acetyl-β-D-hexosaminidase (Peak II in Fig. 2A) gave the same sequential exoglycosidase digestion pattern as the dotted line corresponding to the radioactively labeled peak of Fig. 5B, which suggests structure **23** and structure **24** for the original oligosaccharide.



23 X = GlcNAcOT, R = H
24 X = GlcNAc, R = Galβ→4GlcNAcβ (3.6%)

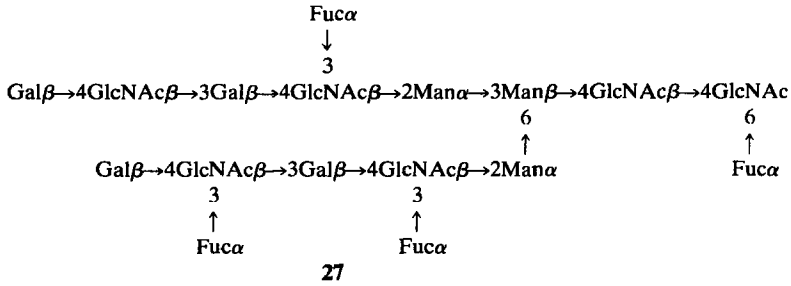
The radioactively labeled peak IV illustrated in Fig. 3A gave the same degradation pattern as the solid-line peak of Fig. 5B by sequential exoglycosidase digestion. Therefore, the resulting oligosaccharide had structure **25**. The original oligosaccharide of fraction **N2-2** had an additional Galβ→4GlcNAcβ→2 group in its outer chain, which suggests structure **26**.

The radioactively labeled fraction **AN1** gave a sequential exoglycosidase digestion pattern similar to that of fraction **N1** (Fig. 4), except that one group each of D-galactose and 2-acetamido-2-deoxy-D-glucose were released by incubation with a mixture of jack bean β-D-galactosidase and jack bean *N*-acetyl-β-D-hexosaminidase (data not shown). These results suggested that a single

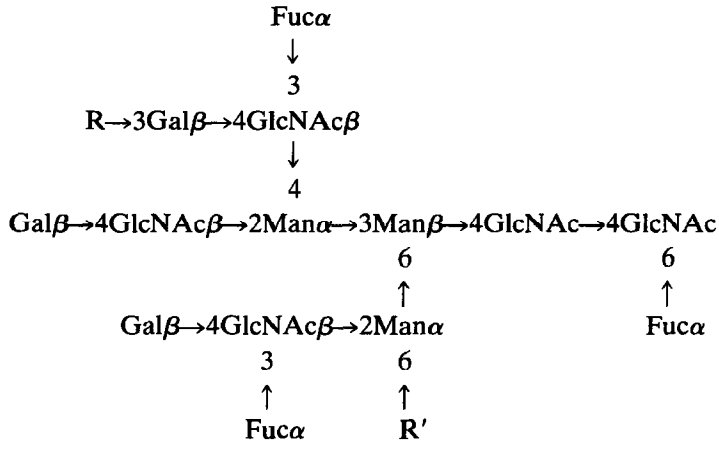


25 X = GlcNAcOT, R = H
26 X = GlcNAc, R = Gal→4[Fucα(∓)→3]GlcNAcβ (2.3%).

Galβ→4GlcNAcβ nonreducing terminal group of the outer chains of oligosaccharides in fraction **AN1** was not fucosylated. As will be described in a subsequent paragraph, this nonfucosylated Galβ→4GlcNAc group is exclusively located on the outer chain linked at O-2 of the Manα→3 residue of the trimannosyl core, thus structures **27–29** for oligosaccharides of fraction **AN1**.



27



28 R = H, R′ = Galβ→4(Fucα→3)GlcNAcβ
29 R = Galβ→4(Fucα→3)GlcNAcβ, R′ = H

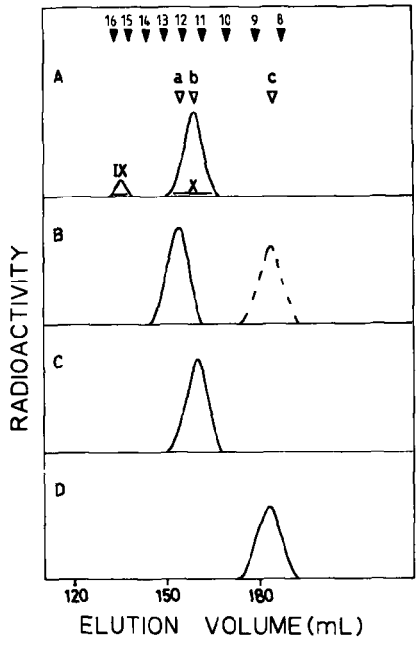
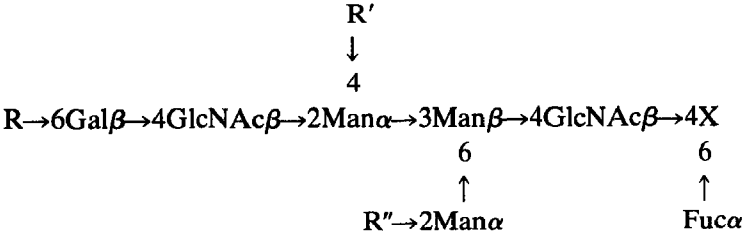


Fig. 6. Sequential exoglycosidase digestion of radioactively labeled fraction **A**. The analytical conditions and black triangles are the same as described in the legend to Fig. 1-II. The white triangles (a, b, and c) indicate the positions where authentic **10**, **11**, and **8** were eluted, respectively. (A) Fraction **A** incubated first with a mixture of almond α-L-fucosidase I, jack bean β-D-galactosidase, and jack bean *N*-acetyl-β-D-hexosaminidase, and then with sialidase; (B) the solid and dotted lines indicate, respectively, the radioactively labeled products obtained from peaks IX and X in panel A by incubation sequentially with diplococcal β-D-galactosidase and diplococcal *N*-acetyl-β-D-hexosaminidase; (C) the peak indicated by the solid line in panel B incubated with almond α-L-fucosidase I; and (D) the radioactively labeled peak in panel C incubated sequentially with diplococcal β-D-galactosidase and diplococcal *N*-acetyl-β-D-hexosaminidase.

Location assignment of the sialyl groups in the acidic oligosaccharides. — As already described, only monosialyl derivatives were found in human milk galactosyltransferase. In order to determine the location of *N*-acetylneuraminyl groups in these oligosaccharides, the following sequential exoglycosidase treatment was performed. A part (5×10^4 c.p.m.) of the radioactively labeled fraction **A** was incubated with a mixture of almond α-L-fucosidase I, jack bean β-D-galactosidase and jack bean *N*-acetyl-β-D-hexosaminidase to remove completely the nonsialylated outer chain components. The glycosidases in the reaction mixture were inactivated by heat treatment, and the resulting radioactively labeled oligosaccharides were desialylated by incubation with sialidase. The radioactively labeled neutral oligosaccharides were separated, by Bio-Gel P-4 column chromatography, into two components that were eluted at the positions of 15.0 and 11.3 glucose units (Fig. 6A). These components, in the molar ratio of 1:9, were named fractions **IX** and **X**, respectively. The radioactively labeled fraction **X** was totally resistant to α-D-mannosidase digestion (data not shown). However, it was completely converted

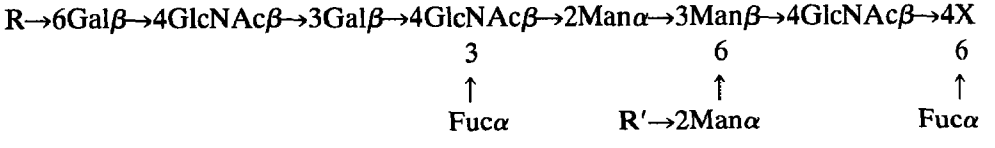
into a radioactively labeled oligosaccharide having the same mobility as that of authentic **8** by incubation with a mixture of diplococcal β-D-galactosidase and diplococcal *N*-acetyl-β-D-hexosaminidase (Fig. 6B, dotted line). Since the mobility of fraction **X** was the same as that of authentic **11**, structure **30** was assumed. An identical structure derives from **21, 22, 28**, and **29**. Therefore these oligosaccharides occur as **31, 32, 35**, and **36** in fraction **A**.



30 X = GlcNAcOT, R = R′ = R″ = H

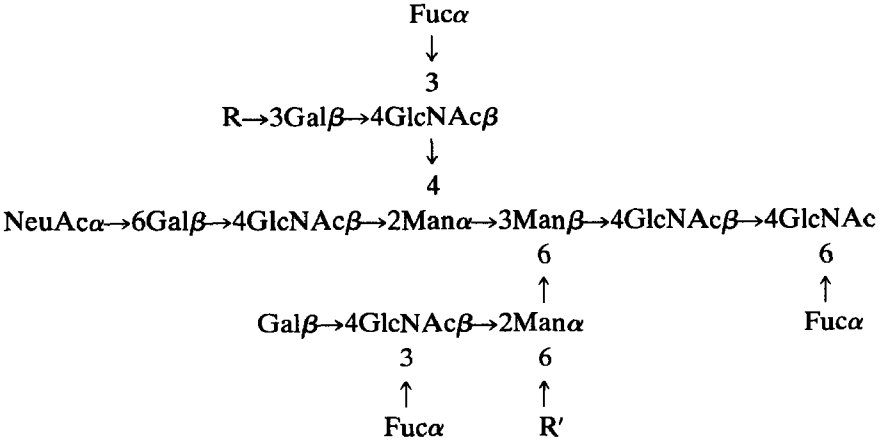
31 X = GlcNAc, R = NeuAcα, R′ = H, R″ = Galβ→4(Fucα→3)GlcNAcβ (30.8%)

32 X = GlcNAc, R = NeuAcα, R′ = R″ = Galβ→4(Fucα→3)GlcNAcβ (19.6%)



33 X = GlcNAcOT, R = R′ = H

34 X = GlcNAc, R = NeuAcα, R′ = Galβ→4(Fucα→3)GlcNAcβ→3Galβ→(Fucα→3)GlcNAcβ (4.2%)



35 R = H, R′ = Galβ→4(Fucα→3)GlcNAcβ (0.7%)

36 R = Galβ→4(Fucα→3)GlcNAcβ, R′ = H (0.7%)

Fraction **IX** was resistant to almond α-L-fucosidase I and α-D-mannosidase digestion. However, it was completely converted into a radioactively labeled oligosaccharide having the same mobility as that of authentic **10** by sequential digestion with diplococcal β-D-galactosidase and diplococcal *N*-acetyl-β-D-hexosaminidase (Fig. 6B, solid line), which removed one group each of D-galactose and 2-acetamido-2-deoxy-D-glucose. The radioactively labeled oligosaccharide shown by the solid line in Fig. 6B released an L-fucosyl group by almond α-L-fucosidase I digestion (Fig. 6C) but was still resistant to α-D-mannosidase digestion (data not shown). When the product illustrated in Fig. 6C was incubated sequentially with diplococcal β-D-galactosidase and diplococcal *N*-acetyl-β-D-hexosaminidase, it released another pair of D-galactose and 2-acetamido-2-deoxy-D-glucose groups, and was converted into a radioactively labeled oligosaccharide having the same mobility as that of authentic **8** (Fig. 6D). These results indicated that the oligosaccharide of fraction **IX** has structure **33**. As undecasaccharide **33** derives from oligosaccharide **27**, it occurs in the sialylated form **34** in fraction **A**.

DISCUSSION

All the sugar chains of the galactosyltransferase reported herein contain structure **8** as their core portion*, but the sugar chains show an extraordinarily high structural multiplicity. Since the galactosyltransferase molecule contains only one asparagine-linked sugar chain, it is heterogeneous in its carbohydrate moiety.

We have recently confirmed that the difference in the total number of acidic sugar chains in one molecule is the molecular basis of the isoforms of rat kidney γ-glutamyltranspeptidase as shown by isoelectric focusing[22]. Is the charge heterogeneity of human milk galactosyltransferase revealed by the same fractionation method[3] also related to the heterogeneity of the carbohydrate components as reported herein?

Two lines of evidence argue against such a relationship: (a) After extensive treatment with sialidase, the galactosyltransferase was still resolved in at least six isoforms[3]. (b) By lectin chromatography on Con A– and lentil lectin–Sepharose column, the galactosyltransferase could be resolved into approximately equal parts of bound and unbound enzyme forms, both of them providing a similar pattern on isoelectric focusing[23].

The sugar chain pattern of human milk galactosyltransferase shows some similarity to the patterns of two other human milk glycoproteins; i.e., lactoferrin[24] and secretory component[25]. The Galβ→4(Fucα→3)GlcNAcβ group is included in the outer chains of all three glycoproteins and the *N*-acetylneuraminic acid group is linked only at O-6 of the D-galactopyranosyl residue. However. prominent structural differences are also found among the sugar chains of these milk glycoproteins, a basic one being in the number of outer chains. In the case of lactoferrin

*GlcNAc instead of GlcNAcOT.

and secretory component, only biantennary sugar chains were observed[24,25]. In contrast, galactosyltransferase contains not only bi-, but also tri- and tetra-antennary oligosaccharide chains. A structural difference was also observed within the biantennary oligosaccharide chains of the three milk glycoproteins. Lactoferrin and secretory component contain structure **6** (GlcNAc instead of GlcNAcOT) and its mono- and di-sialylated forms as major oligosaccharide chains[24,25]. In contrast, galactosyltransferase does not contain these oligosaccharide chains at all. These structural differences found among the sugar chains of three milk glycoproteins might be induced by the difference of the polypeptide structures around the Asn-X-Thr(or Ser) groups to be glycosylated.

The Gal$\beta\rightarrow$4(Fuc$\alpha\rightarrow$3)GlcNAc group is also found in the outer portions of the asparagine-linked sugar chains of other human glycoproteins, such as parotid α-amylase[8] and α_1-acid glycoprotein[26]. Only α_1-acid glycoprotein contain tri- and tetra-antennary oligosaccharide chains with the L-fucose-containing trisaccharide structure. However, in this case, the trisaccharide outer chain is linked at O-4 of the Man$\alpha\rightarrow$3 and O-2 of the Man$\alpha\rightarrow$6 residue of the trimannosyl core. Therefore, the oligosaccharides of fractions **N2-1** (**15**) and **A-2** (**32**), and the tri- and tetra-antennary oligosaccharides in fractions **N1** (**18**) and **A1** (**34**) were found for the first time in a human glycoprotein.

Galactosyltransferase occurs in soluble form also in human serum. Kim *et al.*[27] suggested that most of the serum galactosyltransferase is derived from liver tissue. Yamaguchi and Yoshida[5] purified the human serum galactosyltransferase and analyzed the carbohydrate component. Although only on the basis of monosaccharide composition, they suggested that the enzyme may contain two asparagine-linked sugar chains in one molecule. They also suggested that bi- and tri-antennary complex-type sugar chains are included in this enzyme. One of the most interesting points of their data is that human serum galactosyltransferase does not contain any L-fucose. Therefore, important structural differences are expected to occur between the sugar chains of the two galactosyltransferases. Since organ-specific differences in the sugar chains of γ-glutamyltranspeptidase are well documented[28], it might be interesting to elucidate the sugar chain structures of the human serum or liver galactosyltransferase.

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