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The structure of carbohydrate unit B of porcine thyroglobulin

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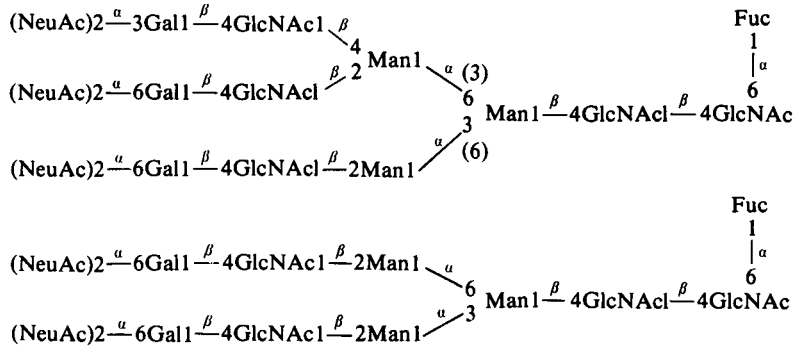
The oligosaccharide fraction was obtained from porcine thyroglobulin by hydrazinolysis. Four fractions of unit B-type oligosaccharides were purified by successive chromatographies on columns of DEAE-cellulose and concanavalin A–Sepharose, and their structures were investigated by the combination of endo- and exo-glycosidase digestions, methylation analysis and Smith degradation. From the results of these studies, the structures of the unit B oligosaccharides were proposed to be as follows:



Thus the glycoprotein was found to have triantennary and biantennary complex-type oligosaccharides as acidic sugar chains. Concerning the triantennary oligosaccharides, the following structural features were shown: (1) the sialic acid residues were not localized on certain specific branches but distributed on all three branches; (2) however, α(2→3)-linked sialic acid residues were exclusively located on the terminal of the branch arising from C-4 of the branching α-mannose residue, whereas α(2→6)-linked sialic acid residues occupied terminals of the other branches; (3) the outer branching α-mannose residue was attached to C-3 or C-6 of an inner branching β-linked mannose residue, and both types were observed to exist.

Thyroglobulin is a major glycoprotein synthesized in the thyroid gland. The carbohydrate moiety of the glycoprotein has been extensively investigated, and it was shown to consist of both high-mannose-type (unit A-type) and complex-type (unit B-type) oligosaccharides being linked to asparagine residues in the peptide moiety in all species studied (Arima *et al.*, 1972). Fukuda & Egami (1971) found triantennary complex-type sugar chains in porcine thyroglobulin and estimated their structures. More recently, Kondo *et al.* (1977) studied the structure of a part of these triantennary oligosaccharides. However, we have found that the oligosaccharides employed in these previous studies were still contaminated by biantennary oligosaccharides and this fact made interpretation of the results difficult. This prompted us to elucidate the structures of all of the unit B-type oligosaccharides purified carefully by ion-exchange chromatography and affinity chromatography on a column of concanavalin A–Sepharose that had been shown to be extremely useful for the separation of biantennary oligosaccharides from triantennary oligosaccharides (Ogata *et al.*, 1975; Krusius *et al.*, 1976). In the present paper, we report the results of this study.

Abbreviations used: GlcNAcol, *N*-acetylglucosaminitol; NeuAc, *N*-acetylneuraminic acid.

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Materials and methods

Isolation of oligosaccharides from porcine thyroglobulin

Porcine thyroglobulin, which was prepared by the method of Ui & Tarutani (1961), was exhaustively digested by the method of Fukuda & Egami (1971). To release oligosaccharide moieties from the glycopeptides thus prepared, hydrazinolysis was carried out by the method described previously (Fukuda *et al.*, 1976). In brief, the dried purified glycopeptides (300mg) were heated at 105°C for 4h in a sealed evacuated tube with 2ml of freshly distilled anhydrous hydrazine containing 20mg of hydrazine sulphate. After evaporation of the hydrazine *in vacuo*, the residue was dried over conc. H_2SO_4 in a vacuum desiccator and then dissolved in a small amount of 4.5M-sodium acetate. After centrifugation at 30000*g* for 10min, the oligosaccharides in the supernatant were *N*-acetylated by the method of Spiro (1972). *N*-Acetylated oligosaccharides were then isolated by subjecting the reaction mixture to gel chromatography on a column (1.5cm × 100cm) of Sephadex G-25 at room temperature.

Fractionation of unit B-type oligosaccharides

The oligosaccharides released by hydrazinolysis were then subjected to ion-exchange chromatography on a DEAE-cellulose column (1.2cm × 25cm) equilibrated with 2mM-Tris/HCl, pH7.4, and the column was eluted with the same buffer. After unbound oligosaccharides were eluted out, acidic unit B-type oligosaccharides were eluted with a linear concentration gradient of NaCl (5–100mM) in the same buffer. Unit B-type oligosaccharides were further fractionated by affinity chromatography on a concanavalin A–Sepharose column. Concanavalin A, purified by the method of Agrawal & Goldstein (1972) from jack-bean meal (Sigma), was coupled to Sepharose 4B as described previously (Matsumoto *et al.*, 1980). The column was first washed with 10mM-sodium acetate buffer, pH6.0, containing 150mM-NaCl, 1mM-$CaCl_2$ and 1mM-$MnCl_2$, and the oligosaccharides bound to the affinity adsorbent were eluted with the same buffer containing 50mM-α-methyl D-mannoside, 150mM-NaCl, 1mM-$CaCl_2$ and 1mM-$MnCl_2$.

Analytical methods

Neutral sugars were assayed by the phenol/H_2SO_4 method (Dubois *et al.*, 1956). Total sialic acid content was measured by the periodate/resorcinol reaction (Jourdian *et al.*, 1971). *N*-Acetylneuraminic acid was identified by g.l.c. on a column (0.3cm × 200cm) of Gas-Chrom Q coated with 2% OV-1, after methanolysis followed by trimethylsilylation by the method of Yu & Ledeen (1970). The carbohydrate composition of the oligosaccharide was analysed by using a gas-liquid chromatograph with a column (0.3cm × 100cm) of 0.05% ECNSS-M; the carbohydrates were chromatographed as alditol acetates after hydrolysis with 2M-HCl at 100°C for 3h.

Glycosidase digestions

β-Galactosidase, β-*N*-acetylhexosaminidase and α-mannosidase were purified from jack-bean meal (Li & Li, 1972). α-L-Fucosidase from *Charonia lampas* and endo-β-*N*-acetylglucosaminidase D from *Diplococcus pneumonea* were purchased from Seikagaku Kogyo (Tokyo, Japan). β-Mannosidase from snail was kindly supplied by Dr. T. Okuyama, Seikagaku Kogyo.

Oligosaccharides were digested at 37°C with glycosidase (0.1–0.5 units) in 0.1ml of the appropriate buffers under a toluene layer for 24–48h followed by heating at 100°C for 3min to terminate the reaction. Then the reaction mixture was passed through small columns of Dowex 50W (X8; H^+ form) and Bio-Rad AG-3 (OH^- form). Digestions with β-galactosidase, β-*N*-acetylhexosaminidase and α-mannosidase were carried out in 50mM-sodium acetate buffer (pH4.0). For digestion with α-L-fucosidase and β-mannosidase, 0.1M-sodium citrate/0.1M-sodium phosphate buffer (pH4.0) containing 0.5M-NaCl was used. Endo-β-*N*-acetylglucosaminidase D digestion was carried out in 0.1M-sodium citrate/0.1M-sodium phosphate buffer (pH6.0).

Gel-permeation chromatography

Gel-permeation chromatography was performed using a high-pressure liquid chromatograph (Jasco Tri-rotor; Japan Spectroscopic Co., Tokyo, Japan) with a column (0.8cm × 100cm) of Bio-Gel P-4 (–400 mesh) at a flow rate of 0.3ml/min as described previously (Tsuji *et al.*, 1980). During operation, the column was maintained at 55°C by a 'water jacket'. Oligomers of glucose and *N*-acetylglucosamine were used as standards. Sugars were monitored with a refractive-index detector (Shodex RI SE-11; Showa Denko, Tokyo, Japan) and a u.v. spectrophotometer (Uvidec 100-II; Jasco, Tokyo, Japan).

Methylation analysis

$NaBH_4$-reduced oligosaccharides were methylated by the method of Hakomori (1964). The permethylated products were purified on a silica-gel column by the method of Liang *et al.* (1979) and subjected to hydrolysis, reduction and acetylation as described by Stellner *et al.* (1973) except that hydrolysis was carried out in 3M-HCl at 80°C for 3h. The alditol acetates of the partially methylated sugars were analysed by g.l.c. on a column (0.3cm × 200cm) of Gas-Chrom Q coated with 2%

OV-1, 3% OV-17 or 3% OV-210. Each peak was identified with a gas chromatograph–mass spectrometer (Shimadzu-LKB model 9000) with a column (0.3 cm × 200 cm) of 1.5% OV-1 on Gas-Chrom Q (80–100 mesh). Conditions for the mass spectrometry were as follows: ion source temperature, 270°C; separator temperature, 260°C; ionizing potential, 70 eV; trap current, 60 μA.

Periodate oxidation

The reduced oligosaccharides were oxidized in 0.5 ml of 50 mM-sodium acetate buffer, pH 4.0, containing 0.05 M-sodium metaperiodate, for 48–96 h at 4°C in the dark. The reaction was terminated by adding 10 μl of ethylene glycol. After 1 h at room temperature, 0.5 ml of 0.1 M-borate buffer, pH 8.0, containing 50 mM-$NaBH_4$ was added and the mixture was kept in the dark for 16 h at 4°C. The excess $NaBH_4$ was destroyed with 1 drop of glacial acetic acid. The oxidized and borohydride-reduced oligosaccharides were isolated by gel filtration on Bio-Gel P-2.

Smith degradation

The periodate-oxidized and borohydride-reduced oligosacharides were hydrolysed with 25 mM-H_2SO_4 at 80°C for 1 h, and the products were isolated by passing the mixture through a column of Dowex AG-3 (200–400 mesh; OH^- form) as described previously (Irimura *et al.*, 1981).

Results

Purification of unit B-type oligosaccharides from porcine thyroglobulin

When *N*-acetylated oligosaccharides released from porcine thyroglobulin glycopeptides by hydrazinolysis were subjected to ion-exchange chromatography on DEAE-cellulose, three major oligosaccharide fractions (UA, UB-I and UB-II) were detected (Fig. 1). Carbohydrate composition analyses showed that fraction UA, which was recovered without absorption, consisted of high-mannose-type oligosaccharides containing mannose and *N*-acetylglucosamine. The structural analysis of these sugar chains is the subject of the preceding paper (Tsuji *et al.*, 1981). Fractions UB-I and UB-II, which emerged with a linear concentration gradient of NaCl, were found to consist of complex-type oligosaccharides containing sialic acid, in addition to galactose, fucose, mannose and *N*-acetylglucosamine, and represented the unit B-type sugar chains.

Both fraction UB-I and fraction UB-II appeared to be heterogeneous, since gel-permeation chromatography of each of these fractions on a column of Bio-Gel P-4 after removal of sialic acids gave rise to two peaks that slightly overlapped each other.

Compositional analysis revealed that the larger oligosaccharide fraction had higher galactose and glucosamine contents than the smaller one (results

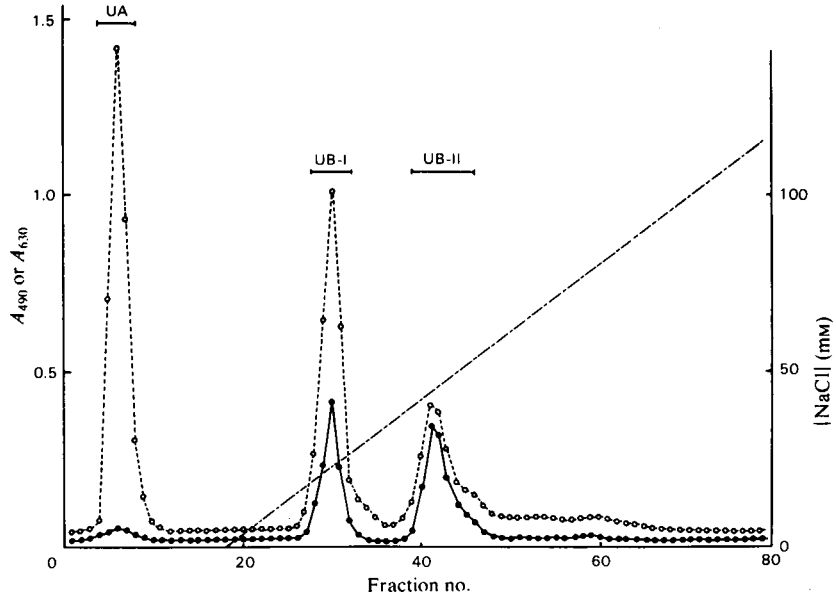


Fig. 1. *Ion-exchange chromatography of oligosaccharides*

The DEAE-cellulose column (1.2 cm × 25 cm) was washed with 2 mM-Tris/HCl, pH 6.0. After eluting the column with the same buffer (fractions 1–18), elution was carried out with 200 ml of a linear gradient of 2 mM-Tris/HCl buffer, pH 6.0, to 2 mM-Tris/HCl buffer, pH 6.0, containing 150 mM-NaCl (fractions 19–80). Each 8.0 ml fraction was analysed for neutral sugar (O) and sialic acid (●). Fractions were pooled as indicated by bars and freeze-dried.

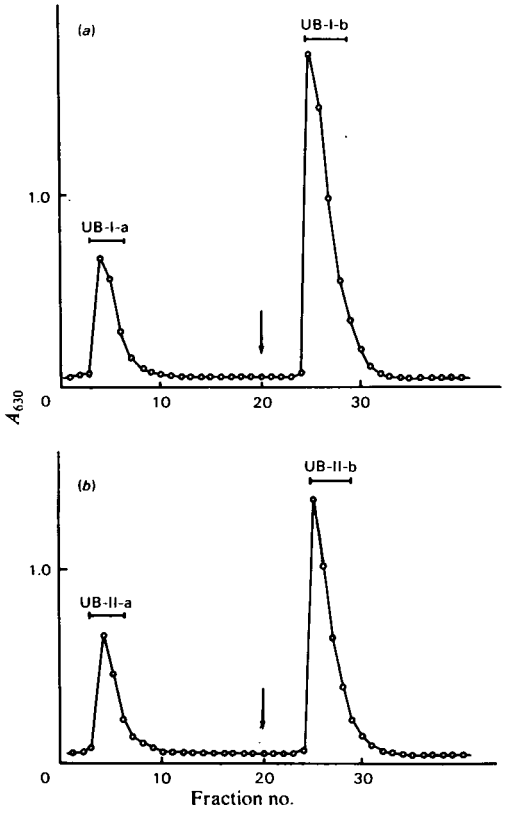


Fig. 2. *Affinity chromatography of the acidic oligosaccharide fractions on a concanavalin A–Sepharose column*

Experimental details are given in the Materials and methods section. Elution was with 10mM-sodium acetate buffer, pH6.0, containing 150mM-NaCl, 1mM-$CaCl_2$ and $MnCl_2$, followed by the same buffer containing 50mM-α-methyl D-mannoside, 150mM-NaCl, 1mM-$CaCl_2$ and $MnCl_2$. The arrow indicates where the buffer was changed. (*a*) Elution profile of UB-I oligosaccharide fraction. (*b*) Elution profile of UB-II oligosaccharide fraction.

not shown). This suggested that the difference in the elution positions of the two peaks from a Bio-Gel P-4 column was due to the difference in the numbers of the branches that were composed of galactose and *N*-acetylglucosamine residues. Therefore, fractions UB-I and UB-II were further fractionated by affinity chromatography on a concanavalin A–Sepharose column. As shown in Fig. 2, fractions UB-I and UB-II each were separated into two fractions. Fractions UB-I-a and UB-II-a emerged at the column volume, whereas the second fractions (UB-I-b and UB-II-b) were obtained after the column was eluted with 50mM-α-methyl D-mannoside. Fractions UB-I-a, UB-I-b, UB-II-a and UB-II-b were separately pooled and their carbohydrate compositions were analysed (Table 1). The results showed that both fraction UB-I-a and fraction UB-I-b contained one sialyl residue, whereas fractions UB-II-a and UB-II-b had two sialyl residues. Fractions UB-I-a and UB-II-a, which were not bound to the concanavalin A–Sepharose column, had 3 galactose and 5 *N*-acetylglucosamine residues, whereas fractions UB-I-b and UB-II-b, which were retained on the concanavalin A–Sepharose column, had 2 galactose and 4 *N*-acetylglucosamine residues. In all the four fractions, the sialic acid was identified as *N*-acetylneuraminic acid.

Sequential exoglycosidase digestion of oligosaccharides

To determine the sequence and anomeric configuration of the sugars, sequential exoglycosidase digestion was performed. After each glycosidase digestion, the reaction mixture was subjected to gel-permeation chromatography on a Bio-Gel P-4 column (Fig. 3) and the carbohydrate composition of the residual oligosaccharide was analysed (Table 2). In these studies, we used asialo-oligosaccharides obtained by mild acid hydrolysis with 0.05M-H_2SO_4 at 80°C for 1h as substrates.

Table 1. *Carbohydrate compositions of oligosaccharides and the products after their periodate oxidation*

	Molar ratio				
Oligosaccharide	Fuc	Man	Gal	GlcNAc	Sialic acid
UB-I-a*	1.0	3.0	3.1	5.2	0.9
UB-I-b*	1.1	3.0	1.9	4.1	1.0
UB-II-a*	0.9	3.0	3.2	5.2	1.8
UB-II-b*	0.9	3.0	2.1	4.2	1.8
After periodate oxidation					
UB-I-a†	0.0	2.0	0.5	5.3	0.0
UB-II-a†	0.0	2.0	0.8	5.1	0.0

* Normalized to 3.0mol of mannose.
† Normalized to 2.0mol of mannose.

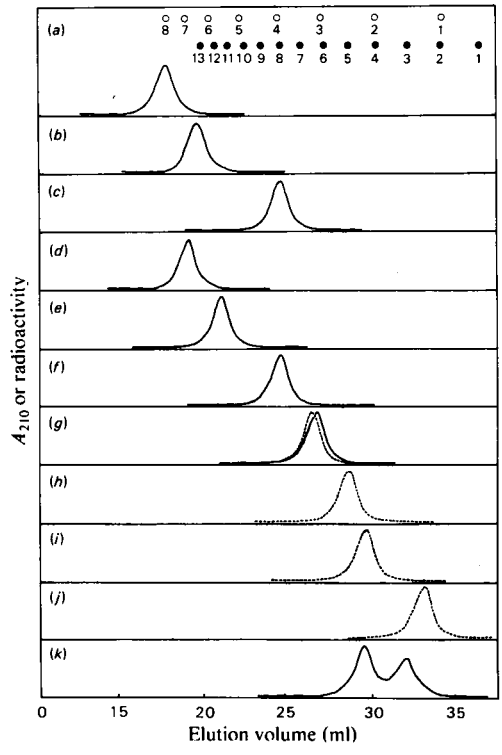


Fig. 3. *Elution profiles of oligosaccharides UB-I-a and UB-I-b after glycosidase digestion on Bio-Gel P-4*
Experimental details are given in the text. Closed circles of glucose oligomers and chitin oligosaccharides, the numbers indicating the number of glucose and *N*-acetylglucosamine units. Sugars were monitored by A_{210} (——) and radioactivity (– – –). (*a*) Asialo UB-I-1 oligosaccharide; (*b*) product of β-galactosidase treatment of (*a*); (*c*) product of β-*N*-acetylhexosaminidase treatment of (*b*); (*d*) asialo UB-I-b oligosaccharide; (*e*) product of β-galactosidase treatment of (*d*); (*f*) product of β-*N*-acetylhexosaminidase treatment of (*e*); (*g*) product of α-mannosidase treatment of (*c*); (*h*) product of β-mannosidase treatment of NaB^3H_4-reduced (*g*); (*i*) product of α-L-fucosidase treatment of (*h*); (*j*) product of β-*N*-acetylhexosaminidase treatment of (*i*), (*k*) products of endo-β-*N*-acetylglucosaminidase D treatment of (*c*).

Asialo-fraction UB-I-a. When asialo-fraction UB-I-a (Fig. 3*a*) was incubated with jack-bean β-galactosidase, three galactose residues were removed (Fig. 3*b*), and three *N*-acetylglucosamine residues were liberated from this digest on incubation with β-*N*-acetylhexosaminidase (Fig. 3*c*). The product released two mannose residues on further digestion with α-mannosidase (Fig. 3*g*). The resulting oligosaccharide, which consisted of one mannose, one fucose and two *N*-acetylglucosamine residues, was then labelled by reduction with NaB^3H_4. β-Mannosidase treatment of the reduced tetrasaccharide resulted in a decrease in size corresponding to one mannose residue (Fig. 3*h*). This digest, shown in Fig. 3(*h*), co-migrated with GlcNAcβ1-4GlcNAcol after α-L-fucosidase treatment (Fig. 3*i*). Subsequent digestion with β-*N*-acetylhexosaminidase gave a radioactive product that co-migrated with GlcNAcol (Fig. 3*j*). When asialo-fraction UB-I-a that had previously been digested with β-galactosidase and then with β-*N*-acetylhexosaminidase was further treated with endo-β-*N*-acetylglucosaminidase D from *Diplococcus pneumonea*, two fragments were obtained (Fig. 3*k*). The lower-molecular-weight fragment was found to consist of fucose and *N*-acetylglucosamine in a ratio of 1:1 and the other had mannose and *N*-acetylglucosamine in a ratio of 3:1 (Table 2).

Asialo-fraction UB-I-b. When asialo-fraction UB-I-b (Fig. 3*d*) was treated with β-galactosidase, two galactose residues were removed (Fig. 3*e*). This digest released two *N*-acetylglucosamine residues on β-*N*-acetylhexosaminidase treatment (Fig. 3*f*). This oligosaccharide component, shown in Fig. 3(*f*), was eluted at the same position as the oligosaccharide obtained by successive digestion of asialo-fraction UB-I-a with β-galactosidase and β-*N*-acetylhexosaminidase (Fig. 3*c*). The sequence of this core hexasaccharide from fraction UB-I-b was found to be identical with that from fraction UB-I-a, since the same results were obtained after α-mannosidase, β-mannosidase, α-L-fucosidase and endo-β-*N*-acetylglucosaminidase D digestions in both cases.

Asialo-fractions UB-II-a and UB-II-b. The sugar sequences of asialo-fractions UB-II-a and UB-II-b were found to be identical with those of asialo-fractions UB-I-a and UB-I-b respectively, on the basis of the results of glycosidase digestions with endoglycosidase and exoglycosidases (results not shown).

From these series of experiments with exoglycosidase and endoglycosidase, the sugar sequences of asialo-fractions UB-I-a and UB-II-a were found to be $(\beta Gal)_3$-$(\beta GlcNAc)_3$-$(\alpha Man)_2$-βMan-$\beta GlcNAc$-(αFuc-)GlcNAc and those of asialo-fractions UB-I-b and UB-II-b were $(\beta Gal)_2$-$(\beta GlcNAc)_2$ - $(\alpha Man)_2$ - βMan - $\beta GlcNAc$ - (αFuc-)GlcNAc.

Structures of asialo-fractions UB-I-a, UB-I-b, UB-II-a and UB-II-b

To determine the location of each glycosidic linkage, $NaBH_4$-reduced oligosaccharides, from which sialic acid had been removed, were subjected to methylation analyses and Smith degradation. The results of methylation analyses of the oligo-

Table 2. *Sequential enzymic digestion and endoglycosidase digestion of unit B-type oligosaccharides*

	Molar ratio			
(*a*) Sequential enzymic digestions	Fuc	Gal	Man	GlcNAc
Asialo-fraction UB-I-a	0.8	3.3	3.0*	5.4
β-Galactosidase	0.9	0.0	3.0*	5.2
Then β-*N*-acetylhexosaminidase	0.9	0.0	3.0*	2.1
Then α-mannosidase	0.8	0.0	1.2	2.0†
Asialo-fraction UB-I-b	0.9	2.3	3.0*	4.3
β-Galactosidase	0.9	0.0	3.0*	4.2
Then β-*N*-acetylhexosaminidase	0.9	0.0	3.0*	2.0
Then α-mannosidase	0.9	0.0	1.1	2.0†
(*b*) Endo-β-*N*-acetylglucosaminidase D digestion of $(Man)_3(GlcNAc)_2Fuc$				
Higher-molecular-weight fragment	0.0	0.0	3.0*	1.0
Lower-molecular-weight fragment	0.9	0.0	0.0	1.0‡

* Normalized to 3.0 mol of mannose.
† Normalized to 2.0 mol of *N*-acetylglucosamine.
‡ Normalized to 1.0 mol of *N*-acetylglucosamine.

Table 3. *Methylation analyses of UB-I-a, UB-I-b, UB-II-a and UB-II-b asialo-oligosaccharides*
Molar ratios are expressed in relation to 2,4-di-*O*-methylmannitol taken as 1.0.

	O-Methyl groups		Molar ratios in oligosaccharides			
Sugar	Number	Position	Asialo-UB-I-a	Asialo-UB-I-b	Asialo-UB-II-a	Asialo-UB-II-b
Fucitol	3	2, 3, 4	0.6	0.7	0.7	0.8
Galactitol	4	2, 3, 4, 6	2.8	1.7	2.6	1.8
Mannitol	3	3, 4, 6	1.1	2.2	1.2	2.2
	2	2, 4	1.0	1.0	1.0	1.0
	2	3, 6	0.8	0.0	0.8	0.0
2-Deoxy-2-*N*-methyl-acetamidoglucitol	3	1, 3, 5	0.7	0.7	0.8	0.7
	2	3, 6	4.4	3.2	4.2	3.2

saccharides of asialo-fractions UB-I-a, UB-I-b, UB-II-a and UB-II-b and their Smith degradation products are shown in Tables 3 and 4.

Asialo-fraction UB-I-a. The results of the methylation study showed that all the fucose and galactose residues constituted non-reducing terminals. Four of the five *N*-acetylglucosamine residues were detected as 2-deoxy-3,6-di-*O*-methyl-2-*N*-methylacetamidoglucitol and the other was obtained as 2-deoxy-1,3,5-tri-*O*-methyl-2-*N*-methylacetamidoglucitol, indicating that the reducing terminal was 4,6-disubstituted *N*-acetylglucosamine. After α-L-fucosidase treatment followed by methylation analysis, 2-deoxy-1,3,5,6-tetra-*O*-methyl-2-*N*-methylacetamidoglucitol was detected instead of the 4,6-disubstituted derivative (results not shown). This result showed that a fucose residue was linked to C-6 of the *N*-acetylglucosamine residue at the reducing terminal. Three mannose residues were detected as 3,4,6-tri-*O*-methylmannitol, 2,4-di-*O*-methylmannitol and 3,6-di-*O*-methylmannitol in a ratio of about 1:1:1. To clarify the branching structure of the three mannose residues, asialo-fraction UB-I-a was subjected to Smith degradation. When the Smith degradation product of asialo-fraction UB-I-a was subjected to gel-permeation chromatography on a Bio-Gel P-4 column, the main peak was observed at a position between $(GlcNAc)_5$ and $(GlcNAc)_6$ (Fig. 4*a*), which consisted of two mannose and four *N*-acetylglucosamine residues (Table 4*a*). Methylation analysis of this product revealed the presence of 3,6-di-*O*-methylmannose, 2,3,4-tri-*O*-methylmannose and 2,4,6-tri-*O*-methylmannose in a molar ratio of about 1:0.5:0.5 (Table 4*b*). This showed that the β-linked mannose residue located at the inner branching point was 3,6-disubstituted and that 2,4-disubstituted mannose constituted the outer branching point. Furthermore, this result indicated

Table 4. *Compositional and methylation analyses of Smith degradation products*

			Molar ratio			
					UB-I-a	
	Smith degradation product of ...		Asialo-UB-I-a	Asialo-UB-I-b	S-I	S-II
(*a*) Compositional analyses						
Fucose			0.0	0.0	0.0	0.0
Mannose			2.0*	1.0†	2.0*	2.0*
Galactose			0.0	0.0	1.0	0.3
N-Acetylglucosamine			4.1	2.4	4.4	4.5
	O-Methyl groups					
	Number	Position				
(*b*) Methylation analyses						
Galactitol	4	2, 3, 4, 6	0.0		0.6	0.1
Mannitol	3	2, 3, 4	0.5		0.5	0.5
	3	2, 4, 6	0.5		0.5	0.5
	2	2, 4	0.1		0.2	0.1
	2	3, 6	0.7		0.7	0.8
2-Deoxy-2-*N*-methylacetamido-	4	1, 3, 5, 6	0.8		0.8	0.9
glucitol	3	3, 4, 6	2.4		1.6	2.3
	2	3, 6	1.0‡		2.0§	1.0‡

* Normalized to 2.0 mol of mannose.
† Normalized to 1.0 mol of mannose.
‡ Normalized to 1.0 mol of 2-deoxy-3,6-di-*O*-methyl-2-*N*-methylacetamidoglucitol.
§ Normalized to 2.0 mol of 2-deoxy-3,6-di-*O*-methyl-2-*N*-methylacetamidoglucitol.

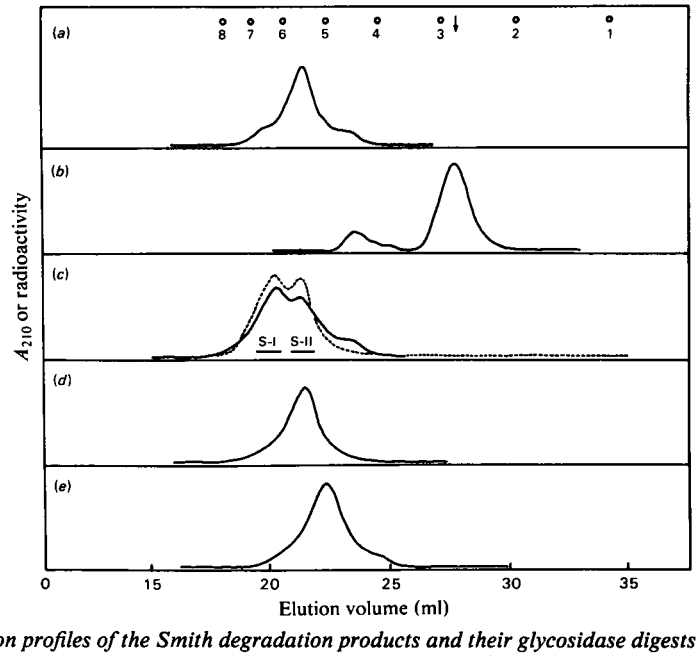
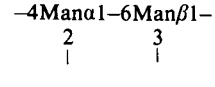


Fig. 4. *Elution profiles of the Smith degradation products and their glycosidase digests on Bio-Gel P-4*
Experimental details are given in the Materials and methods section. Open circles indicate the elution positions of chitin oligosaccharides, and the numbers indicate the numbers of *N*-acetylglucosamine units. The arrow indicates the elution position of the standard sugar; Manβ1-4GlcNAcβ1-4GlcNAcol. Sugars were monitored by A_{210} (——) and radioactivity (– – – –). (*a*) Smith degradation products of asialo-oligosaccharide UB-I-a; (*b*) Smith degradation products of asialo-oligosaccharide UB-I-b; (*c*) Smith degradation products of oligosaccharide UB-I-a; (*d*) product of β-galactosidase treatment peak S-I in (*c*); (*e*) product of β-*N*-acetylhexosaminidase treatment of peak S-I in (*c*).

that the asialo-fraction UB-I-a consisted of two types of oligosaccharides, which contained



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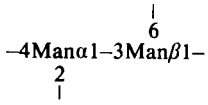


groups respectively in equal amounts.

Asialo-fraction UB-II-a. The results of methylation analysis (Table 3) and Smith degradation (results not shown) for asialo-fraction UB-II-a were found to be identical with those for fraction UB-I-a.

The data thus establish the structure of both asialo-fraction UB-I-a and asialo-fraction UB-II-a to be as shown in Fig. 5(*a*).

Asialo-fraction UB-I-b. The results of methylation studies are shown in Table 3. As in the cases of asialo-fraction UB-I-a and asialo-fraction UB-II-a fucose and galactose residues were found to constitute non-reducing terminals. Three of the four *N*-acetylglucosamine residues were substituted on C-4 and the other was shown to be a reducing terminal substituted at C-4 and 6. After α-L-fucosidase treatment followed by methylation analysis, the reducing terminal *N*-acetylglucosamine residue was converted into the 4-monosubstituted derivative, indicating that the fucose residue was linked to C-6 of the reducing terminal *N*-acetylglucosamine residue (results not shown). Two of the three mannose residues were detected as 3,4,6-tri-*O*-methyl derivatives and the other as 2,4-di-*O*-methylmannitol. To elucidate the position of the 3,6-disubstituted branching mannose residue, asialo-fraction UB-I-b was subjected to Smith degradation. When the product was applied to a Bio-Gel P-4 column (Fig. 4*b*), the major peak was eluted at the same position as Manβ1-4GlcNAcβ1-4GlcNAcol on a Bio-Gel P-4 column. This fraction was found to consist of one mannose and two *N*-acetyl-

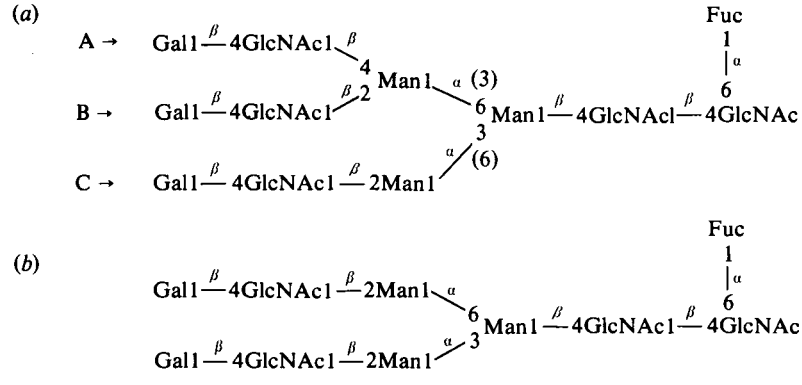


Fig. 5. *Proposed structures of asialo-oligosaccharides UB-I-a, UB-II-a, UB-I-b and UB-II-b*

Table 5. *Methylation analyses of oligosaccharides UB-I-a, UB-I-b, UB-II-a and UB-II-b and their glycosidase digests*

	O-Methyl groups		Molar ratio						
Sugar	Number	Position	UB-I-a	UB-I-b	UB-II-a	UB-II-b	UB-I-a (GH*)	UB-I-b (GH*)	UB-II-a (GH*)
Fucitol	3	2, 3, 4	0.6	0.7	0.7	0.8	0.6	0.7	0.7
Galactitol	4	2, 3, 4, 6	2.3	0.8	0.9	—	—	—	—
	3	2, 3, 4	0.7	0.9	1.2	2.1	0.7	0.8	1.1
	3	2, 4, 6	0.3	—	0.7	—	0.3	—	0.7
Mannitol	4	2, 3, 4, 6	—	—	—	—	1.1	0.9	0.3
	3	2, 3, 6	—	—	—	—	0.4	—	0.4
	3	3, 4, 6	0.8	2.1	1.1	2.0	0.6	0.9	1.0
	2	2, 4	1.0	1.0	1.0	1.0	1.0	1.0	1.0
	2	3, 6	0.8	—	0.8	—	—	—	0.3
2-Deoxy-2-*N*-methylacetamidoglucitol	3	1, 3, 5	0.7	0.7	0.9	0.8	1.0	0.7	0.9
	2	3, 6	3.8	3.1	4.3	3.2	2.3	2.2	3.2

* Treatment with a mixture of β-galactosidase and β-*N*-acetylhexosaminidase.

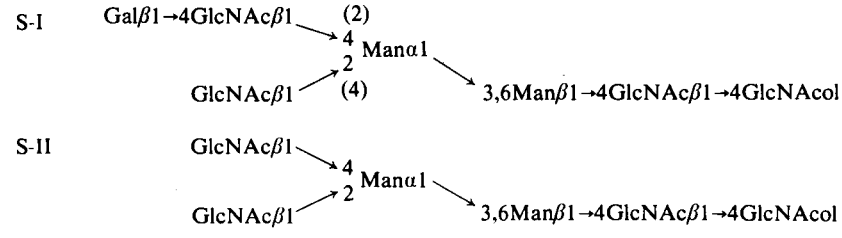
glucosamine residues (Table 4). This result indicates that the branching mannose is located in the inner portion of fraction UB-I-b.

Asialo-fraction UB-II-b. Methylation analysis and Smith degradation of asialo-fraction UB-II-b gave the same results as those of asialo-fraction UB-I-b (Table 3).

The data so far establish the structure of both asialo-fraction UB-I-b and asialo-fraction UB-II-b to be as shown in Fig. 5(*b*).

Location of the sialic acid residues

Fraction UB-I-a. This was found to have one sialyl residue linked to C-3 or C-6 of the galactose residue, since the methylation of this oligosaccharide gave 2,3,4,6-tetra-*O*-methylgalactitol, 2,3,4-tri-*O*-methylgalactitol and 2,4,6-tri-*O*-methylgalactitol in a molar ratio of 2.3:0.7:0.3 (Table 5). When fraction UB-I-a was completely digested with a mixture of β-galactosidase and β-*N*-acetylhexosaminidase and the digest was then subjected to methylation analysis, 4-substituted mannose, 2-substituted mannose, 3,6-disubstituted mannose and non-reducing terminal mannose were detected in a molar ratio of 0.4:0.6:1.0:1.1 (Table 5). The fact that these various mannose derivatives were detected indicates that a sialic acid residue is located at any one of the distal galactose residues on the three sugar chains. The proportion of oligosaccharide UB-I-a that had a sialic acid residue at position A in Fig. 5(*a*) was calculated from the molar ratio of 4-substituted mannose and estimated to be about 40%. On the other hand, about 60% of oligosaccharide UB-I-a appeared to have a sialic acid residue at position B or C (see Fig. 5). This estimation was supported by the following experiment using a concanavalin A–Sepharose column that required at least two α-mannose residues with a free hydroxy group at C-3, -4 and -6 for oligosaccharides to be retained. A part of fraction UB-I-a, which has a sialic acid residue at position B or C, should be retained by the column after treatment with a mixture of β-galactosidase and β-*N*-acetylhexosaminidase. Actually, when the digest of NaB^3H_4-reduced fraction UB-I-a with a mixture of these two glycosidases was applied to a concanavalin A–Sepharose column, 55% of the total radioactivity was bound to the column and eluted with 50 mM-α-methyl D-mannoside. This value was consistent with the previous estimation from the results of the methylation studies. The same experiment was performed with an unlabelled sample, and the fractions thus obtained were subjected to methylation analysis. The bound fraction gave 2,3,4-tri-*O*-methylgalactitol, but no 2,4,6-tri-*O*-methylgalactitol, whereas in the case of the unbound fraction 2,4,6-tri-*O*-methylgalactitol but no 2,3,4-tri-*O*-methylgalactitol was detected (results not shown). These results clearly indicate that the α(2→3)-linked sialic acid residue is located on the branch arising from C-4 of a branching α-mannose residue (position A in Fig. 5*a*) and that the α(2→6)-linked sialic acid residue is located on the branch arising from C-2 of the branching on unbranching α-mannose residue (position B or C in Fig. 5*a*). The selective localization of α(2→3)- and α(2→6)-linked sialyl residues was confirmed by the combination of Smith degradation, β-*N*-acetylhexosaminidase digestion and methylation analysis. When Smith degradation products of fraction UB-I-a were subjected to gel-permeation chromatography on a Bio-Gel P-4 column, a higher-molecular-weight peak (S-I) was detected besides the peak (S-II) whose elution position was identical with that of the main product of asialo-fraction UB-I-a (Fig. 4*c*). Peak S-I was found to contain one galactose residue in addition to two mannose and four *N*-acetylglucosamine residues. On the basis of the data from methylation analysis (Table 4), peaks S-I and S-II were determined to be as follows:



Although more than one terminal *N*-acetylglucosamine residue was detected in the methylation analysis of fraction S-I, it could be due to contamination from fraction S-II. The presence of an additional galactose residue in peak S-I was confirmed by the fact that peak S-I was eluted at the same position as peak S-II after digestion with β-galactosidase (Fig. 4*d*). This galactose residue, which was resistant to periodic oxidation, should be the one originally substituted at C-3 by a sialic acid residue in fraction UB-I-a. Methylation analysis of the digest of peak S-I with β-*N*-acetylhexosaminidase (Fig. 4*e*) revealed that the mannose-bearing substituents at both C-2 and C-4 (3,6-di-*O*-methylmannitol) were converted

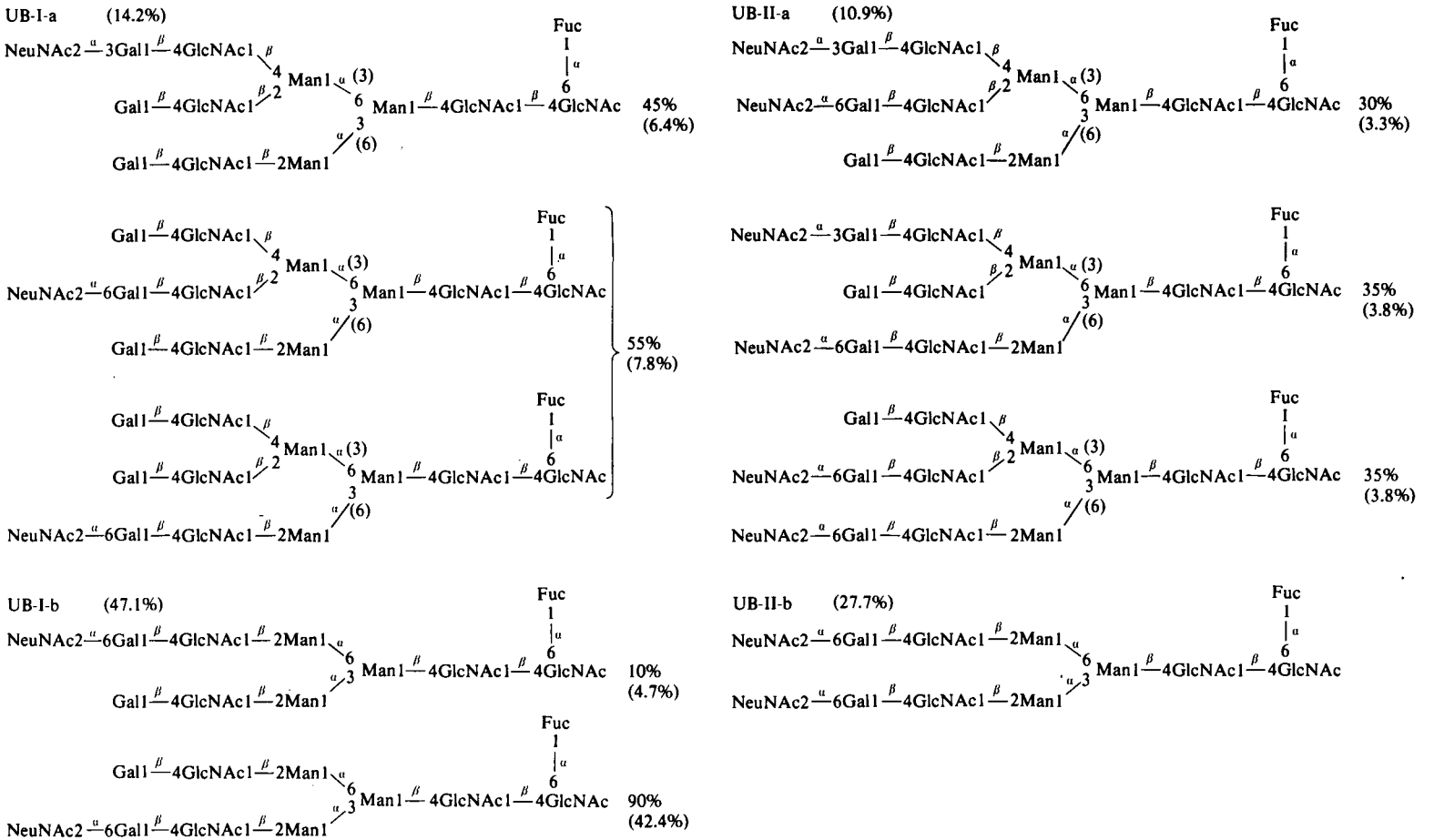


Fig. 6. *Proposed structures of oligosaccharides UB-I-a, UB-I-b, UB-II-a and UB-II-b*

Values in parentheses represent the amounts of respective oligosaccharide fractions present expressed as a percentage of the total unit B-type oligosaccharides, which were obtained by DEAE-cellulose ion-exchange chromatography. Abbreviation used: NeuNAc, *N*-acetylneuraminate.

by this enzymic treatment into a mannose residue having only one substituent at C-4 (2,3,6-tri-*O*-methylmannitol). This again indicated that the β(2→3)-linked sialic acid residue is linked to the galactose residue in the branch arising from C-4 of the branching α-mannose residue.

Fraction UB-II-a. This was found to have two sialyl residues linked to C-3 or C-6 of the galactose residue, since it gave 2,3,4,6-tetra-*O*-methylgalactitol, 2,3,4-tri-*O*-methylgalactitol and 2,4,6-tri-*O*-methylgalactitol in a ratio of 0.9:1.2:0.7 after methylation. When fraction UB-II-a, treated with a mixture of β-galactosidase and β-*N*-acetylhexosaminidase, was subjected to methylation analysis, various mannose derivatives such as 4-substituted, 2-substituted, 2,4-disubstituted, 3,6-disubstituted, and non-reducing terminal mannose were detected (Table 5). This indicated that two sialic acid residues exist in any two terminal positions of the three branches. The proportion of the oligosaccharides that have sialic acid residues at both positions A and B (Fig. 5*a*) was calculated from the molar ratio of the terminal mannose residue or the 2,4-disubstituted mannose derivative and estimated to be about 30%.

The proportion of the oligosaccharide that is substituted by sialic acid residues at both positions A and C was estimated to be about 35% from the molar ratio of the 4-substituted mannose derivative. The rest, 35%, had sialic acid residues at both positions B and C. These estimations were supported by the subsequent experiments using concanavalin A–Sepharose affinity chromatography. When the digest of NaB^3H_4-reduced fraction UB-II-a with a mixture of β-galactosidase and β-*N*-acetylhexosaminidase was applied to a concanavalin A–Sepharose column, about 35% of the total radioactivity was bound to the column and eluted with 50 mM-α-methyl D-mannoside. This result shows that a part of fraction UB-II-a (35%) has sialic acid residues at positions B and C, and becomes capable of being bound to a column of concanavalin A–Sepharose after treatment with a mixture of the two glycosidases. This value was consistent with the previous estimation from the results of the methylation analysis. Furthermore, these two fractions from the concanavalin A-affinity chromatography were subjected to methylation analysis. The bound fraction gave only 2,3,4-tri-*O*-methylgalactitol, no 2,4,6-tri-*O*-methylgalactitol, whereas the unbound fraction gave both 2,4,6-tri-*O*-methyl- and 2,3,4-tri-*O*-methyl-galactitol (results not shown). This indicates that the α(2→3)-linked sialic acid residue does not exist on the branch arising from C-2 of either the branching or the unbranching α-mannose residue (position B or C in Fig. 5*a*). The localization of the α(2→3)-linked sialic acid residue was confirmed also by Smith degradation followed by β-*N*-acetylhexosaminidase digestion and methylation analysis. Smith degradation of fraction UB-II-a produced fragments S-I and S-II as in the case of fraction UB-I-a. Methylation analysis of fragment S-I after treatment with β-*N*-acetylhexosaminidase gave 2,3,6-tri-*O*-methylmannitol but no 3,4,6-tri-*O*-methylmannitol, indicating that the α(2→3)-linked sialic acid residue is localized exclusively at the non-reducing terminal of the branch arising from C-4 of the branching α-mannose residue.

Fraction UB-I-b. This was found to have one sialyl residue linked to C-6 of the penultimate galactose residue, since methylation analysis gave 2,3,4,6-tetra-*O*-methylgalactitol and 2,3,4-tri-*O*-methylgalactitol in an equal molar ratio (Table 5). Then we examined which of the two galactose residues was substituted by use of endo-β-*N*-acetylglucosaminidase D (Tai *et al.*, 1975) as follows. NaB^3H_4-reduced fraction UB-I-b was digested with a mixture of β-galactosidase and β-*N*-acetylhexosaminidase. After inactivation of the two glycosidases by heating at 100°C for 3 min, sialidase was added and the mixture was incubated at 37°C for 16 h. The radioactive product was analysed on a Bio-Gel P-4 column to ensure the digestion was complete, and then incubated with endo-β-*N*-acetylglucosaminidase D. Then the products were again analysed on a Bio-Gel P-4 column, 10% of the radioactivity was recovered as Fuc-GlcNAcol and the rest was eluted at the original position. The oligosaccharide resistant to the enzyme showed no change in the elution position from the column after repeated treatment with the endo enzyme. On the basis of the specificity of endo-β-*N*-acetylglucosaminidase D, 90% of fractions UB-I-b was found to have a sialic acid residue on the branch originating from C-3 of the β-linked mannose.

Fraction UB-II-b. The methylation analysis shown in Table 4 indicated that two galactose residues were substituted by sialyl residues at C-6.

On the basis of the series of experiments described above, we propose the structures of the unit B-type oligosaccharides from porcine thyroglobulin to be as shown in Fig. 6.

Discussion

In the present paper, we propose the structures of unit B-type oligosaccharides of porcine thyroglobulin to be as shown in Fig. 6. They can be classified into two groups according to the number of branches attached to the Manα1-6(Manα1-3)Manβ1-4GlcNAcβ1-4(Fucα1-6)GlcNAc core structure. One group is the so-called triantennary sugar chains (UB-I-a and UB-II-a), and the other the so-called biantennary sugar chain (UB-I-b and

UB-II-b). They could be well separated by affinity chromatography on a concanavalin A–Sepharose column as reported by Krusius *et al.* (1976). Affinity chromatography using such immobilized lectins seemed to be a useful tool for the fractionation of oligosaccharides and glycopeptides. Triantennary complex-type sugar chains have been found in several glycoproteins, for example, calf fetuin (Nilsson *et al.*, 1979; Baenziger & Fiete, 1979), vesicular-stomatitis-virus-G protein (Reading *et al.*, 1978), thyroxine-binding globulin (Zinn *et al.*, 1978) and α_1-proteinase inhibitor (Hodges *et al.*, 1979; Mega *et al.*, 1980). The structures of the triantennary complex-type oligosaccharides of porcine thyroglobulin are of interest in several points when they are compared with those of other triantennary sugar chains. The first is the mode of branching at mannose residues. Unit B triantennary oligosaccharides contain both

$$\begin{array}{l} -4\text{Man}\alpha 1-6\text{Man}\beta 1- \\ \quad\;\; 2 \quad\;\; 3 \\ \quad\;\; | \quad\;\; | \end{array}$$

and

$$\begin{array}{l} \quad\quad\quad\quad\;\; | \\ \quad\quad\quad\quad\;\; 6 \\ -4\text{Man}\alpha 1-3\text{Man}\beta 1- \\ \quad\;\; 2 \\ \quad\;\; | \end{array}$$

types. In the case of fetuin, Baenziger & Fiete (1979) reported that this glycoprotein contained only the former type, whereas Nilsson *et al.* (1979) observed only the latter type in the same glycoprotein. Although the reason for this discrepancy is not clear, these observations, including ours, suggest that a variety of *N*-acetylglucosaminyltransferases is involved in the formation of these branches. The second point is the heterogeneity of the location and the linkage of sialic acid residues. Fractions UB-I-a and UB-II-a consist of isomers that are different only in the location of *N*-acetylneuraminic acid residues. Furthermore, the α(2→3)-linked sialyl residues are exclusively located on the branch arising from C-4 of the branching α-mannose residue, whereas the α(2→6)-linked sialyl residues occupy the other branches from C-2 of the branching or unbranching α-mannose residue. This suggests that at least two sialyltransferases [i.e. α(2→3) sialyltransferase and α(2→6) sialyltransferase] are involved in the synthesis of triantennary oligosaccharides, and that the specificity of each enzyme directs not only a β-galactose residue but also the sugar sequence including mannose as a whole. However, these sialyltransferases appear to have different specificities depending on the tissues or the species, since the locations of α(2→3)- or α(2→6)-linked sialic acid residues in the fetuin glycopeptides and in the vesicular-stomatitis-virus-G protein have been reported to be different from that in the present study.

In this paper, we present also the structures of the biantennary oligosaccharides (UB-I-b and UB-II-b) that have one or two α(2→6)-linked *N*-acetylneuraminic acid residues, and these structures have been found in other sources (Kornfeld & Kornfeld, 1976).

In a previous paper (Kondo *et al.*, 1977), we reported the structures of the triantennary unit B-type glycopeptides. The reported structures contained branching mannose residues of only the

$$\begin{array}{l} -6\text{Man}\alpha 1- \\ \quad\;\; 3 \\ \quad\;\; | \end{array}$$

type and no α(2→3)-linked sialic acid residues. The discrepancy with the results in the present paper may be explained by the fact that, in the previous study, the triantennary complex-type glycopeptides employed for the structural analysis were still contaminated by biantennary glycopeptides, which led to misinterpretation of the structure of triantennary glycopeptides.

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