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Elongation of both branches of biantennary backbones of oligo-(*N*-acetyllactosamino)glycans by human serum (1→3)-*N*-acetyl-β-D-glucosaminyltransferase

Anja Vilkman, Ritva Niemelä, Leena Penttilä, Jari Helin, Anne Leppänen, Antti Seppo, Hannu Maaheimo, Sari Lusa, and Ossi Renkonen*

Institute of Biotechnology, University of Helsinki, Valimotie 7, 00380 Helsinki, and Department of Biochemistry, Unioninkatu 35, 00170 Helsinki (Finland)

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ABSTRACT

Partial reactions catalyzed by a (1→3)-*N*-acetyl-β-D-glucosaminyltransferase (EC 2.4.1.149), known to be present in human serum, were studied by use of biantennary "backbone" saccharides of oligo-*N*-acetyllactosamine-type as acceptors. Incubation of the radiolabeled blood-group I-active hexasaccharide, β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc-(1→3)-[β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc-(1→6)]-β-D-Gal*p*-(1→4)-D-GlcNAc (**1**) and UDP-GlcNAc with serum gave first a transient 1:1 mixture of two isomeric heptasaccharides, β-D-Glc*p*NAc-(1→3)-β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc-(1→3)-[β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc-(1→6)]-β-D-Gal*p*-(1→4)-D-GlcNAc (**2**) and β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc-(1→3)-[β-D-Glc*p*NAc-(1→3)-β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc-(1→6)]-β-D-Gal*p*-(1→4)-D-GlcNAc (**3**), showing that both branches of **1** react equally well. The two heptasaccharides reacted further in the incubation mixture to form the radiolabeled octasaccharide, β-D-Glc*p*NAc-(1→3)-β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc-(1→3)-[β-D-Glc*p*NAc-(1→3)-β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc-(1→6)]-β-D-Gal*p*-(1→4)-D-GlcNAc (**4**); during this second reaction, the composition of the heptasaccharide mixture remained unchanged, indicating that **2** and **3** reacted at approximately equal rates. The heptasaccharides **2** and **3** could not be separated from each other, but they could be detected, identified, and quantitatively determined by stepwise enzymic degradations. Partial (1→3)-*N*-acetyl-β-D-glucosaminylation reactions, carried out with another acceptor, the branched pentasaccharide, β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc-(1→3)-[β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc-(1→6)]-β-D-Gal (**11**), revealed that it reacted also equally well at both branches. Moreover, the initially formed hexasaccharides, β-D-Glc*p*NAc-(1→3)-β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc-(1→3)-[β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc-(1→6)]-β-D-Gal (**12**) and β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc-(1→3)-[β-D-Glc*p*NAc-(1→3)-β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc-(1→6)]-β-D-Gal (**13**), were converted into the heptasaccharide, β-D-Glc*p*NAc-(1→3)-β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc-(1→3)-[β-D-Glc*p*NAc-(1→3)-β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc-(1→6)]-β-D-Gal (**14**), at nearly equal rates. Radiolabeled tetrasaccharides, β-D-Glc*p*NAc-(1→3)-[β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc-(1→6)]-β-D-Gal (**17**) and β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc-(1→3)-[β-D-Glc*p*NAc-(1→6)]-β-D-Gal (**18**), were *N*-acetyl-D-glucosaminylated almost completely by UDP-GlcNAc in serum-catalyzed reactions. These results suggested that, the (1→6) branches of oligo (*N*-acetyllactosamino)glycan backbones can be elongated as well as the (1→3) branches, thus allowing the build up of a large number of different oligo-(*N*-acetyllactosamino)glycans of branched nature by enzymic *in vitro* synthesis.

* To whom correspondence should be addressed at Institute of Biotechnology, University of Helsinki, Valimotie 7, 00380 Helsinki, Finland (Telefax 358-0-434 6028).

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INTRODUCTION

The growth of branched oligo-*N*-acetyllactosaminoglycans is still rather poorly understood, although the individual enzymes involved, (1→4)-D-β-galactosyltransferase (EC 2.4.1.38), (1→3)-*N*-acetyl-β-D-glucosaminyltransferase (EC 2.4.1.149), and (1→6)-*N*-acetyl-β-D-glucosaminyltransferase (EC 2.4.1.148), are well known. *In vitro* biosynthesis of pure saccharides with the aid of branch-specific partial reactions should be possible, as well as the construction of useful mixtures of glycans through randomly proceeding partial reactions. The branched pentasaccharide, βGal→4βGlcNAc→3(βGal→4βGlcNAc→6)Gal* (**11**), has been shown to be an acceptor for (1→3)-*N*-acetyl-β-D-glucosaminyltransferases of human serum[1] and Novikoff tumor cell ascites fluid[2]. The branched tetrasaccharide, βGal→4βGlcNAc→3(βGlcNAc→6)Gal (**18**), is reportedly a poor acceptor for the serum enzyme[1], but a good acceptor for the Novikoff tumor cell enzyme[2]. The latter enzyme acted at the same rate on **18** and the isomeric tetrasaccharide, βGlcNAc→3(βGal→4βGlcNAc→6)Gal (**17**)[2]. These data suggested that the Novikoff tumor enzyme may act randomly at the (1→3) and (1→6) branches, whereas the serum enzyme may act preferentially at the (1→6) branch. The present report describes the structural analysis of the products obtained by partial *N*-acetyl-D-glucosaminylation reactions catalyzed by the serum enzyme using different biantennary acceptors. The results indicated that the serum enzyme acts equally well at the (1→3) and the (1→6) branches of the biantennary *N*-acetyllactosaminoglycans that were tested.

EXPERIMENTAL

Materials. — UDP-D-galactose, UDP-2-acetamido-2-deoxy-D-glucose, and unlabeled markers, D-lyxose, D-threose, D-galactose, lactose, maltotriose, maltotetraose, maltopentaose, maltoheptaose, and β-D-Glc*p*NAc-(1→6)-D-Gal, were from Sigma (St. Louis, MO, USA). UDP-D-[6-^{3}H]Gal was from Amersham (Buckinghampshire, U.K.). *N,N',N''*-Triacetylchitotriitol, *N,N',N'',N'''*-tetraacetylchitotetraitol, *N,N',N'',N''',N''''*-pentaacetylchitopentaitol, and *N,N',N'',N''',N'''',N'''''*-hexaacetylchitohexaitol were prepared by treating the corresponding reducing saccharides (Seikagaku, Tokyo, Japan) with $NaBH_4$. Synthetic radiolabeled saccharides, βGlcNAc→3(βGlcNAc→6)[U-^{14}C]Gal and βGlcNAc→3(βGlcNAc→6)β[U-^{14}C]Gal→4GlcNAc, were prepared as described earlier[3]. Metabolically labeled teratocarcinoma saccharides, β[U-^{14}C]-Gal→4GlcNAc, βGlcNAc→6[6-^{3}H]Gal, βGlcNAc→3β[U-^{14}C]Gal→4GlcNAc, and β[U-^{14}C]GlcNAc→3βGal→4GlcNAc, were obtained as described earlier[4]. βGlcNAc→3(βGal→4βGlcNAc→6)[U-^{14}C]Gal (**17**) was obtained in 92% purity by partial galactosylation of βGlcNAc→3(βGlcNAc→6)[U-^{14}C]Gal according to Blanken *et al.*[5]; its paper-chromatographic mobility is given in Table I. The isomeric tetrasaccharide, βGal→4βGlcNAc→3(βGlcNAc→6)[U-^{14}C]Gal (**18**), was obtained from a partial

*For the systematic and abbreviated nomenclature of oligosaccharides, see chart 1.

Chart 1. Systematic and abbreviated structures of the oligosaccharides synthesized in the present experiments

Systematic structures of oligosaccharides		Abbreviated structures of oligosaccharides
βD-Galp-(1->4)-βD-GlcpNAc 1 ↓ 6 βD-Galp-(1->4)-βD-GlcpNAc-(1->3)-βD-Galp-(1->4)-D-GlcNAc	1	βGal->4βGlcNAc ↘ 6 3 βGal->4GlcNAc ↗ βGal->4βGlcNAc
βD-Galp-(1->4)-βD-GlcpNAc 1 ↓ 6 βD-GlcpNAc-(1->3)-βD-Galp-(1->4)-βD-GlcpNAc-(1->3)-βD-Galp-(1->4)-D-GlcNAc	2	βGal->4βGlcNAc ↘ 6 3 βGal->4GlcNAc ↗ βGlcNAc 3βGal->4βGlcNAc
βD-GlcpNAc-(1->3)-βD-Galp-(1->4)-βD-GlcpNAc 1 ↓ 6 βD-Galp-(1->4)-βD-GlcpNAc-(1->3)-βD-Galp-(1->4)-D-GlcNAc	3	βGlcNAc 3βGal->4βGlcNAc ↘ 6 3 βGal->4GlcNAc ↗ βGal->4βGlcNAc
βD-GlcpNAc-(1->3)-βD-Galp-(1->4)-βD-GlcpNAc 1 ↓ 6 βD-GlcpNAc-(1->3)-βD-Galp-(1->4)-βD-GlcpNAc-(1->3)-βD-Galp-(1->4)-D-GlcNAc	4	βGlcNAc 3βGal->4βGlcNAc ↘ 6 3 βGal->4GlcNAc ↗ βGlcNAc 3βGal->4βGlcNAc
βD-GlcpNAc 1 ↓ 6 βD-GlcpNAc-(1->3)-βD-Galp-(1->4)-βD-GlcpNAc-(1->3)-βD-Galp-(1->4)-D-GlcNAc	5	βGlcNAc ↘ 6 3 βGal->4GlcNAc ↗ βGlcNAc 3βGal->4βGlcNAc
βD-GlcpNAc-(1->3)-βD-Galp-(1->4)-βD-GlcpNAc 1 ↓ 6 βD-GlcpNAc-(1->3)-βD-Galp-(1->4)-D-GlcNAc	6	βGlcNAc 3βGal->4βGlcNAc ↘ 6 3 βGal->4GlcNAc ↗ βGlcNAc
βD-Galp-(1->4)-βD-GlcpNAc 1 ↓ 6 βD-GlcpNAc-(1->3)-βD-Galp-(1->4)-D-GlcNAc	7	βGal->4βGlcNAc ↘ 6 3 βGal->4GlcNAc ↗ βGlcNAc
βD-GlcpNAc 1 ↓ 6 βD-Galp-(1->4)-βD-GlcpNAc-(1->3)-βD-Galp-(1->4)-D-GlcNAc	8	βGlcNAc ↘ 6 3 βGal->4GlcNAc ↗ βGal->4βGlcNAc
βD-Galp-(1->4)-βD-GlcpNAc-(1->6)-βD-Galp-(1->4)-D-GlcNAc	9	βGal->4βGlcNAc ↘ 6 βGal->4GlcNAc
βD-Galp-(1->4)-βD-GlcpNAc-(1->3)-βD-Galp-(1->4)-D-GlcNAc	10	βGal->4βGlcNAc ↗ 3 βGal->4GlcNAc
βD-Galp-(1->4)-βD-GlcpNAc 1 ↓ 6 βD-Galp-(1->4)-βD-GlcpNAc-(1->3)-βD-Gal	11	βGal->4βGlcNAc ↘ 6 3 Gal ↗ βGal->4βGlcNAc
βD-Galp-(1->4)-βD-GlcpNAc 1 ↓ 6 βD-GlcpNAc-(1->3)-βD-Galp-(1->4)-βD-GlcpNAc-(1->3)-βD-Gal	12	βGal->4βGlcNAc ↘ 6 3 Gal ↗ βGlcNAc 3βGal->4βGlcNAc
βD-GlcpNAc-(1->3)-βD-Galp-(1->4)-βD-GlcpNAc 1 ↓ 6 βD-Galp-(1->4)-βD-GlcpNAc-(1->3)-βD-Gal	13	βGlcNAc 3βGal->4βGlcNAc ↘ 6 3 Gal ↗ βGal->4βGlcNAc
βD-GlcpNAc-(1->3)-βD-Galp-(1->4)-βD-GlcpNAc 1 ↓ 6 βD-GlcpNAc-(1->3)-βD-Galp-(1->4)-βD-GlcpNAc-(1->3)-βD-Gal	14	βGlcNAc 3βGal->4βGlcNAc ↘ 6 3 Gal ↗ βGlcNAc 3βGal->4βGlcNAc
βD-GlcpNAc-(1->3)-βD-Galp-(1->4)-βD-GlcpNAc 1 ↓ 6 βD-GlcpNAc-(1->3)-βD-Gal	15	βGlcNAc 3βGal->4βGlcNAc ↘ 6 3 Gal ↗ βGlcNAc
βD-GlcpNAc 1 ↓ 6 βD-GlcpNAc-(1->3)-βD-Galp-(1->4)-βD-GlcpNAc-(1->3)-βD-Gal	16	βGlcNAc ↘ 6 3 Gal ↗ βGlcNAc 3βGal->4βGlcNAc
βD-Galp-(1->4)-βD-GlcpNAc 1 ↓ 6 βD-GlcpNAc-(1->3)-βD-Gal	17	βGal->4βGlcNAc ↘ 6 3 Gal ↗ βGlcNAc
βD-GlcpNAc 1 ↓ 6 βD-Galp-(1->4)-βD-GlcpNAc-(1->3)-βD-Gal	18	βGlcNAc ↘ 6 3 Gal ↗ βGal->4βGlcNAc
βD-Galp-(1->4)-βD-GlcpNAc-(1->6)-βD-Gal	19	βGal->4βGlcNAc ↘ 6 Gal
βD-Galp-(1->4)-βD-GlcpNAc-(1->3)-βD-Gal	20	βGal->4βGlcNAc ↗ 3 Gal

TABLE I

Paper chromatographic mobilities[a] of oligosaccharides **1–20**; synthesized in the present experiments

Oligo-saccharide	*Mobilities*				*Oligo-saccharide*	*Mobilities*			
	R_{MT}	R_{MTet}	R_{MP}	R_{MH}		R_{MT}	R_{MTet}	R_{MP}	R_{MH}
1			0.72	1.48	**11**			0.81	1.58
2			0.49	1.02	**12**			0.58	1.16
3			0.49	1.02	**13**			0.58	1.16
4			0.32	0.66	**14**			0.41	0.82
5			0.91	1.91	**15**			0.97	1.94
6			0.91	1.91	**16**			0.97	1.95
7		0.77	1.24		**17**		0.91	1.39	
8		0.77	1.24		**18**		0.91	1.39	
9	0.68	1.09			**19**	0.78	1.22		
10	0.80	1.29			**20**	0.95	1.49		

[a] R_{MT}, R_{MTet}, R_{MP}, and R_{MH} give the mobilities of the oligosaccharides in relation to maltotriose, maltotetraose, maltopentaose, and maltoheptaose in Solvent *A*, respectively.

E. coli β-D-galactosidase digest of βGal→4βGlcNAc→3(βGal→4βGlcNAc→6)[U-^{14}C]Gal[6]; its paper-chromatographic mobility is given in Table I.

Glycosyltransferase reactions. — (1→4)-β-D-Galactosyltransferase reactions were performed by incubating the labeled acceptor saccharides with UDP-Gal or UDP-[6-^{3}H]Gal and *N*-acetyllactosamine synthase (EC 2.4.1.38) from bovine milk (Sigma), essentially as described by Brew *et al.*[7]. (1→3)-*N*-Acetyl-β-D-glucosaminyltransferase reactions were carried out, essentially as described earlier[3], by incubating the labeled acceptor saccharides with UDP-GlcNAc and human serum, which contains[1,8–10] *N*-acetyllactosaminide (1→3)-*N*-acetyl-β-D-glucosaminyltransferase activity (EC 2.4.1.149). The incubation times varied from 14 to 120 h.

Periodate oxidation. — Periodate oxidation of the disaccharide, βGlcNAc→3[6-^{3}H]Gal, was performed as described earlier[11]. Briefly, the dry, labeled saccharide was cleaved with 15mM $NaIO_4$ in Hough's sodium acetate buffer, pH 3.6 (ref. 12), for 16 h at room temperature. The oxidation product was desalted, then cleaved with M HCl (4 h, 100°), and finally deacidified by ion exchange. The hydrolyzate was analyzed by paper chromatography.

Chromatographic methods. — Chromatography on agarose-bound wheat germ agglutinin (WGA) was performed as described earlier[4]. The affinity of saccharides is presented as a difference, $\triangle = V_e - V_o$, where V_e is the peak fraction of the saccharide and V_o is the peak fraction of D-galactose. Descending paper chromatography was performed with Whatman No. 3 Chr paper and the upper phase of 4:1:5 (v/v) butanol–acetic acid–water (solvent *A*), or 10:1:2 (v/v) butanol–ethanol–water (solvent *E*). The radioactivity in paper chromatograms was analyzed as described earlier[11], but Opti-Scint from Wallac (Turku, Finland) was used as the scintillant. H.p.l.c. was performed with system I of Blanken *et al.*[13].

Exoglycosidase digestions. — Digestions with jack bean *N*-acetyl-β-D-hexosaminidase (EC 3.2.1.30) (Sigma) were carried out in a reaction mixture (45 μL) containing the radiolabeled substrate, the enzyme (150 mU), 0.05M sodium citrate (pH 4.0), and D-galactono-1,4-lactone (10.5 mg/mL). The mixture was incubated for 6–16 h at 37°. Under these conditions, the enzyme cleaved β[U-^{14}C]GlcNAc→3βGal→4GlcNAc completely, but β[U-^{14}C]Gal→4GlcNAc remained intact. Cleavages with β-D-galactosidase (EC 3.2.1.23) from *Diplococcus pneumoniae* (Boehringer, Mannheim, Germany) and from jack bean (Sigma) were carried out as described earlier[11].

Endo-β-D-galactosidase digestions. — Digestion with *Bacillus fragilis* endo-β-D-galactosidase (EC 3.2.1.103) (Boehringer) was carried out in a reaction mixture (40 μL) containing the enzyme (250 mU/mL), 50 mM sodium acetate buffer, pH 5.8, bovine serum albumin (0.2 mg/mL), and NaN_3 (0.5 mg/mL). The mixture was incubated for 24 h at 37°. Under these conditions, the enzyme cleaved βGlcNAc→3β[U-^{14}C]-Gal→4GlcNAc completely.

Desalting. — All enzyme reactions were terminated by heating the reaction mixtures for 3 min at 100°, after which they were desalted by filtration in water through Dowex AG-1 (AcO^-) and Dowex AG-50 (H^+) ion-exchange resins.

RESULTS

Enzymic in vitro *synthesis and characterization of the radiolabeled hexasaccharide, βGal→4βGlcNAc→3(βGal→4βGlcNAc→6)βGal→4GlcNAc* (**1**). — The hexasaccharides, βGal→4βGlcNAc→3(βGal→4βGlcNAc→6)β[U-^{14}C]Gal→4GlcNAc ([^{14}C-]**1**) and β[6-^{3}H]Gal→4βGlcNAc→3(β[6-^{3}H]Gal→4βGlcNAc→6)β[U-^{14}C]Gal→4GlcNAc ([^{3}H]-**1**) were synthesized by incubating βGlcNAc→3(βGlcNAc→6)β[U-^{14}C]-Gal→4GlcNAc with (1→4)β-D-galactosyltransferase and UDP-D-galactose or UDP-D-[6-^{3}H]galactose, respectively. Under the reaction conditions chosen, the acceptors were rapidly exhausted and the fully galactosylated products were formed. Paper chromatography of the hexasaccharide products (Table I), the tetrasaccharide primer[3], and the monogalactosylated primers[6] confirmed that two galactose units were linked to each primer tetrasaccharide. Digestion of the hexasaccharide [^{14}C]-**1** with *D. pneumoniae* β-D-galactosidase gave the original tetrasaccharide (83.5% yield), as revealed by paper chromatography (data not shown). This confirmed that both D-galactose units of the hexasaccharide were β-(1→4)-linked[14].

Incubation of the radiolabeled hexasaccharide **1** *and UDP-GlcNAc with human serum; isolation of a heptasaccharide fraction.* — Incubation of [^{14}C]-**1** and UDP-GlcNAc with human serum gave a mixture that was separated into three components by h.p.l.c. (Fig. 1A). The fastest peak, "hexa", migrated like the original hexasaccharide. The experiments described below showed that the second peak, "hepta", contained the heptasaccharides, βGlcNAc→3βGal→4βGlcNAc→3(βGal→4βGlcNAc→6)β[U-^{14}C]-Gal→4GlcNAc ([^{14}C]-**2**) and βGal→4βGlcNAc→3(βGlcNAc→3βGal→4βGlcNAc→6)β[U-^{14}C]Gal→4GlcNAc ([^{14}C]-**3**). The peak "octa" of Fig. 1A, in turn, was shown to represent the octasaccharide, βGlcNAc→3βGal→4βGlcNAc→3(βGlcNAc→3βGal→

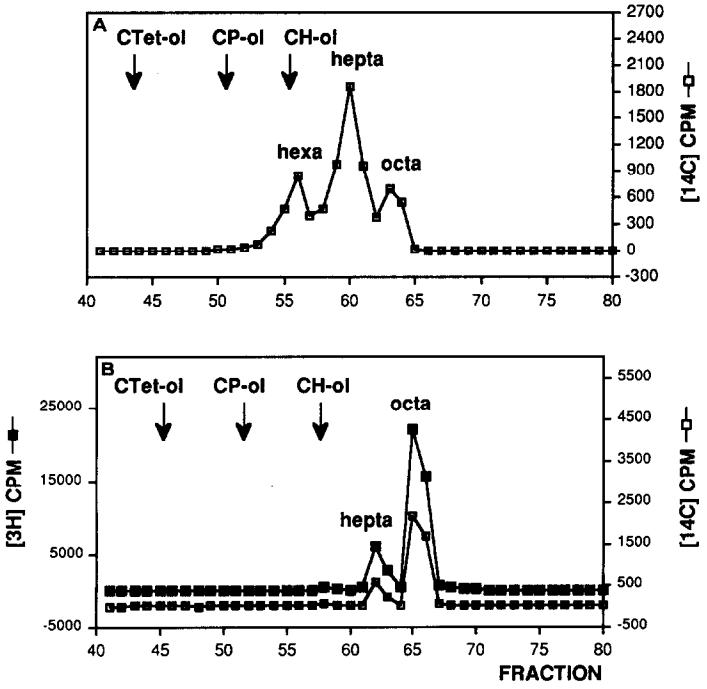
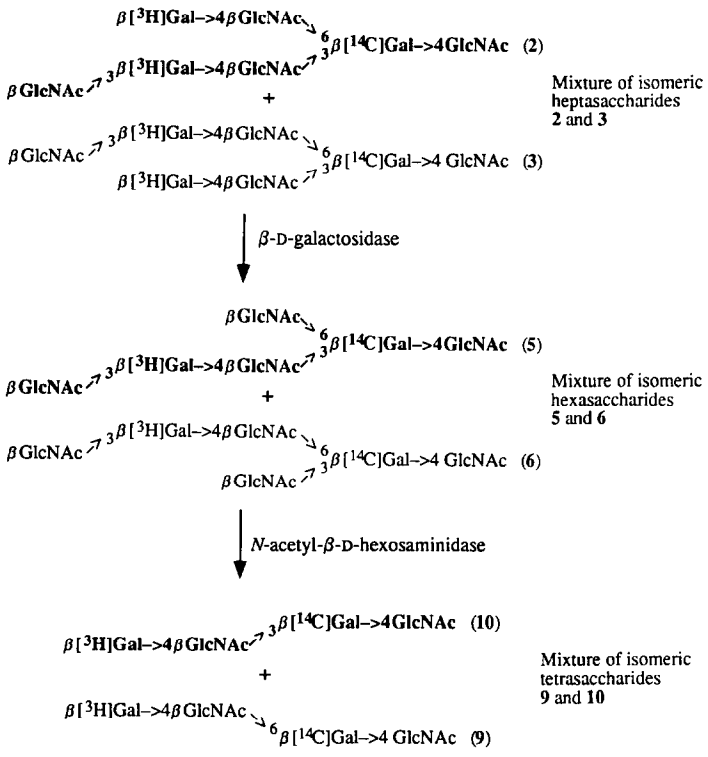


Fig. 1. H.p.l.c. separation of the products from (1→3)-*N*-acetyl-β-D-glucosaminyltransferase reactions: (A) Products from βGal→4βGlcNAc→3(βGal→4βGlcNAc→6)β[U-^{14}C]Gal→4GlcNAc ([^{14}C]-**1**). (B) Products from β[6-^{3}H]Gal→4βGlcNAc→3(β[6-^{3}H]Gal→4βGlcNAc→6)β[U-^{14}C]Gal→4GlcNAc ([^{3}H]-**1**). The desalted reaction mixtures were applied to an h.p.l.c. column of Lichrosorb-NH_2 (particle size, 5 μm; E. Merck, Darmstadt, F.R.G.). The oligosaccharides were eluted first with a mobile phase consisting of acetonitrile (4 vols.) and 15 mM potassium phosphate, pH 5.2 (1 vol.); after 30 min, a linear gradient was applied by increasing the amount of the buffer at a rate of 0.5% /min. The flow rate was 2 mL/min and 2-mL fractions were collected. Fractions 40–80 were analyzed for radioactivity by liquid scintillation counting. The peak labeled "hexa" represents the substrate hexasaccharide in panel (A); in the experiment illustrated in panel (B), the peak "hexa" represents ~ 0.5% of total radioactivity and is not visible. The peaks labeled "hepta" represent mixtures of the radiolabeled heptasaccharides βGlcNAc→3βGal→4βGlcNAc→3(βGal→4βGlcNAc→6)βGal→4GlcNAc (**2**) and βGal→4βGlcNAc→3(βGlcNAc→3Gal→4βGlcNAc→6)βGal→4GlcNAc (**3**). The peaks labeled "octa" represent the radiolabeled octasaccharide βGlcNAc→3βGal→4βGlcNAc→3(βGlcNAc→3βGal→4βGlcNAc→6)βGal→4GlcNAc (**4**). Arrows marked CTet-ol, CP-ol, and CH-ol show the positions of reduced, *N*-acetylated chitotetraose, chitopentaose, and chitohexaose, respectively.

4βGlcNAc→6)β[U-^{14}C]Gal→4GlcNAc ([^{14}C]-**4**) (see below). The relative amounts of the three peaks suggested that 47% of the acceptor sites of the hexasaccharide had reacted in this particular *N*-acetylglucosaminylation experiment.

In another experiment, prolonged incubation of [^{3}H]-**1** and UDP-GlcNAc with serum gave a mixture that proved to contain the heptasaccharides, βGlcNAc→3β[6-^{3}H]Gal→4βGlcNAc→3(β[6-^{3}H]Gal→4βGlcNAc→6)β[U-^{14}C]Gal→4GlcNAc ([^{3}H]-**2**) and β[6-^{3}H]Gal→4βGlcNAc→3(βGlcNAc→3β[6-^{3}H]Gal→4βGlcNAc→6)β[U-^{14}C]-Gal→4GlcNAc ([^{3}H]-**3**), and the octasaccharide, βGlcNAc→3β[6-^{3}H]Gal→4βGlc-

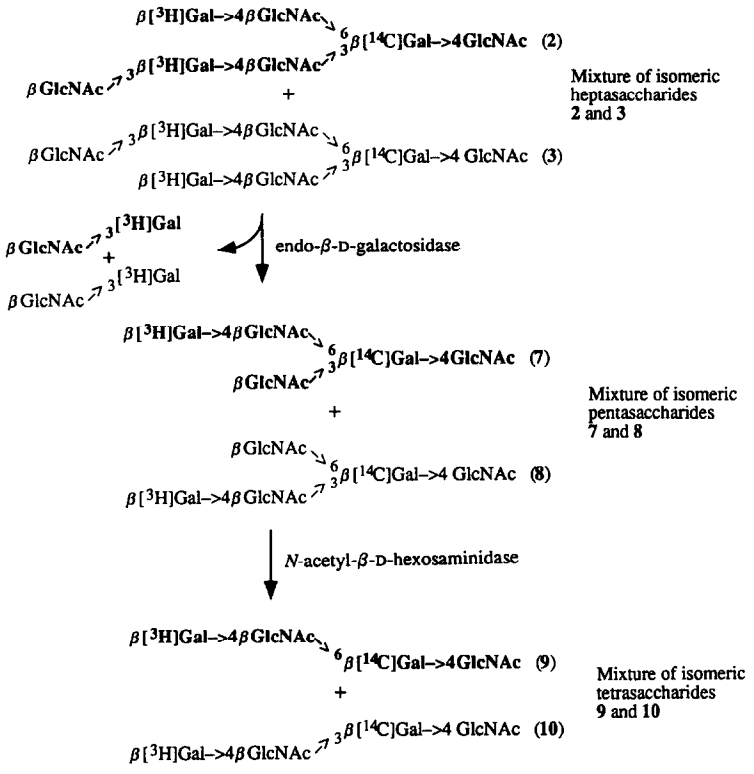


Scheme 1. Outline of sequential cleavage of the mixture of branched heptasaccharides [^{3}H]-**2** and [^{3}H]-**3** with β-D-galactosidase and *N*-acetyl-β-D-hexosaminidase. This process eliminates three monosaccharide units, removing totally yhe nonelongated branches of the heptasaccharides. One of the isomeric substrate heptasaccharides (**2**), and the hexa- (**5**) and tetra-saccharide (**10**) derived from it, are depicted with a bold type of font; this notation should make it clear that **10** is derived from **2**, while **9** is derived from **3**.

NAc→3(βGlcNAc→3β[6-^{3}H]Gal→4βGlcNAc→6)β[U-^{14}C]Gal→4GlcNAc ([^{3}H]-**4**). They were separated from each other by h.p.l.c. (Fig. 1B). In this particular *N*-acetylglucosaminylation experiment, 90% of the acceptor sites of the hexasaccharide [^{3}H]-**1** had reacted.

When digested with jack bean *N*-acetyl-β-D-hexosaminidase, the mixture of [^{14}C]-**2** and [^{14}C]-**3** released the original hexasaccharide [^{14}C]-**1** (data not shown). This confirmed the presence of nonreducing β-linked D-GlcNAc groups in both heptasaccharides.

Compositional analysis of the heptasaccharide fractions. — So far, the heptasaccharides **2** and **3** could not be separated from each other by any chromatographic method (See Fig. 1 and Table I). Thus, the compositions of the heptasaccharide fractions, shown in Fig. 1, were analyzed by sequential enzymic digestion with β-D-galactosidase and *N*-acetyl-β-D-hexosaminidase (see Scheme 1). The resulting linear tetrasaccharides, βGal→4βGlcNAc→6βGal→4GlcNAc (**9**) and βGal→4βGlcNAc→



Scheme 2. Outline of sequential cleavage of the mixture of the branched heptasaccharides [^{3}H]-**2** and [^{3}H]-**3** with endo-β-D-galactosidase and *N*-acetyl-β-D-hexosaminidase. This process eliminates the elongated branches from the heptasaccharides; two types of fonts are used as in scheme 1, showing that here **9** is derived from **2**, while **10** is derived from **3**.

3βGal→4GlcNAc (**10**), were separated from each other by paper chromatography[11]. In another experiment, the mixed heptasaccharides **2** and **3** were cleaved sequentially with endo-β-D-galactosidase and *N*-acetyl-β-D-hexosaminidase (see Scheme 2), whereafter the resulting mixture of tetrasaccharides **9** and **10** was again analyzed by paper chromatography. The identities of tetrasaccharides **9** and **10**, obtained in this experiment, were confirmed by WGA-agarose chromatography.

It is worth noting that the reactions described in Scheme 1 remove the non-elongated branches of the heptasaccharides, whereas the reactions described in Scheme 2 remove the elongated branches. Hence, the two schemes should give well controlled results when used together. The actual experiments are described below in a stepwise manner.

*Digestion of the heptasaccharide mixture ([^{3}H]-**2** and [^{3}H]-**3**) with β-D-galactosidase.* — The heptasaccharide fraction of Fig. 1B originated from a late-stage reaction. Hence, it contained largely molecules that reacted slowly in the

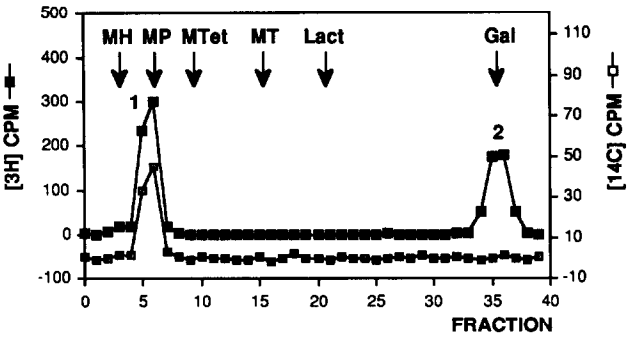


Fig. 2. Paper chromatogram of a *D. pneumoniae* β-D-galactosidase digest of the peak "hepta" from Fig. 1B. The desalted products were irrigated with solvent *A* for 64 h, and the dried chromatogram was counted for radioactivity. Peak 1, hexasaccharides βGlcNAc→3β[6-^{3}H]Gal→4βGlcNAc→3(βGlcNAc→6)β[U-^{14}C]Gal→4GlcNAc ([^{3}H]-**5**) and βGlcNAc→3(βGlcNAc→3β[6-^{3}H]Gal→4βGlcNAc→6)β[U-^{14}C]Gal→4GlcNAc ([^{3}H]-**6**). Peak 2, D-[6-^{3}H]Gal. The arrows marked MH, MP, MTet, MT, Lact, and Gal show the positions of maltoheptaose, maltopentaose, maltotetraose, maltotriose, lactose, and galactose, respectively.

heptasaccharide-to-octasaccharide transformation, and its composition reflects largely the branch specificity of the second *N*-acetylglucosaminylation reaction.

Exhaustive treatment with *D. pneumoniae* β-D-galactosidase released half of [^{3}H]galactose labeled units from the heptasaccharide mixture [^{3}H]-**2** and [^{3}H]-**3** (Fig. 2, Peak 2). A hexasaccharide fraction was also generated; it contained half of the [^{3}H]galactose and all of the [^{14}C]galactose of the original heptasaccharide mixture (Fig. 2, Peak 1). The hexasaccharide fraction proved to be a mixture of βGlcNAc→3β[6-^{3}H]Gal→4βGlcNAc→3(βGlcNAc→6)β[U-^{14}C]Gal→4GlcNAc ([^{3}H]-**5**) and βGlcNAc→3(βGlcNAc→3β[6-^{3}H]Gal→4βGlcNAc→6)β[U-^{14}C]Gal→4GlcNAc ([^{3}H]-**6**) (see below). This hexasaccharide mixture, representing $(GlcNAc)_4Gal_2$ saccharides, migrated faster in paper chromatography than the hexasaccharide **1**, which is a $(GlcNAc)_3Gal_3$ saccharide (See Table I).

These results, combined with the results of the *N*-acetyl-β-D-hexosaminidase degradation of the heptasaccharide mixture of [^{14}C]-**2** and [^{14}C]-**3** (see above), established firmly that the peaks "hepta" of Figs. 1A and 1B consisted of biantennary heptasaccharides that carried one galactosyl and one 2-acetamido-2-deoxyglucosyl group at the nonreducing ends.

Characterization of the hexasaccharide fraction released by β-D-galactosidase from the heptasaccharide fraction [^{3}H]-2 and [^{3}H]-3. — The hexasaccharide fraction from Fig. 2 (Peak 1), supposed to contain the hexasaccharides [^{3}H]-**5** and [^{3}H]-**6**, was further treated with *N*-acetyl-β-D-hexosaminidase. Paper chromatography of the digest revealed equimolar amounts of the tetrasaccharides, β[6-^{3}H]Gal→4βGlcNAc→6β[U-^{14}C]Gal→4GlcNAc ([^{3}H]-**9**) and β[6-^{3}H]Gal→4βGlcNAc→3β[U-^{14}C]Gal→4GlcNAc ([^{3}H]-**10**), having *R* values identical to those of authentic βGal→4βGlcNAc→6βGal→4GlcNAc and βGal→4βGlcNAc→3βGal→4GlcNAc[11] (See Fig. 3A and Table

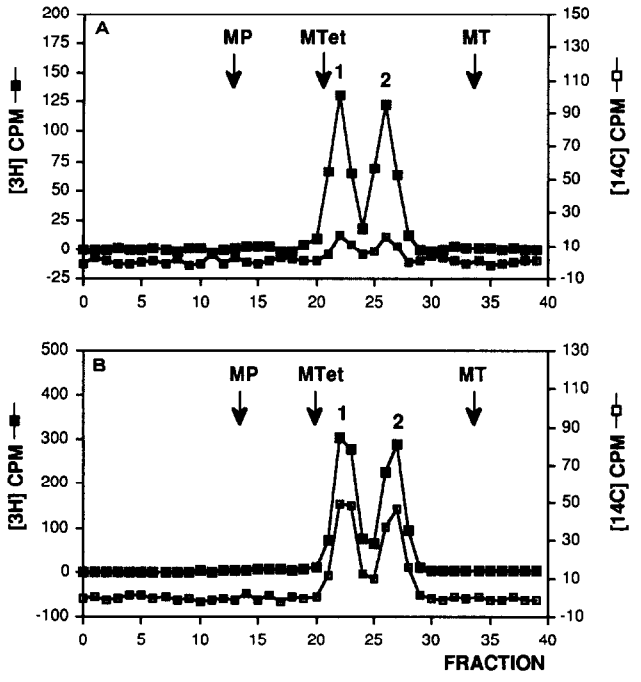


Fig. 3. Paper chromatograms of jack bean *N*-acetyl-β-D-hexosaminidase digests: (A) Digest of Peak 1 of Fig. 2. (B) Digest of Peak 1 of Fig. 4A. The desalted products were irrigated with solvent *A* for 136 and 142 h, respectively, and the dried chromatograms were counted for radioactivity. Peak 1, tetrasaccharide β[6-^{3}H]Gal→4βGlcNAc→6β[U-^{14}C]Gal→4GlcNAc ([^{3}H]-**9**). Peak 2, tetrasaccharide β[6-^{3}H]Gal→4βGlcNAc→3β[U-^{14}C]Gal→4GlcNAc ([^{3}H]-**10**). The arrows marked MP, MTet, and MT show the positions of maltopentaose, maltotetraose, and maltotriose, respectively.

I). Thus, the hexasaccharide fraction was a 1:1 mixture of glycans [^{3}H]-**5** and [^{3}H]-**6**, and the original heptasaccharide mixture was an equimolar mixture of components [^{3}H]-**2** and [^{3}H]-**3**.

*Digestion of the mixture of heptasaccharides [^{3}H]-**2** and [^{3}H]-**3** with endo-β-D-galactosidase; presence of βGlcNAc→3[6-^{3}H]Gal sequences in the heptasaccharides.* — Treatment of the heptasaccharide fraction from Fig. 1B, containing heptasaccharides [^{3}H]-**2** and [^{3}H]-**3**, with endo-β-D-galactosidase from *B. fragilis* gave a mixture that was resolved, in paper chromatography, into two peaks of equal ^{3}H-radioactivity (Fig. 4A). The faster component of Fig. 4A (peak 2) proved to be a disaccharide. It was completely cleaved by jack bean *N*-acetyl-β-D-hexosaminidase, releasing [^{3}H]galactose, which was identified by paper chromatography (data not shown). The [^{3}H]disaccharide migrated like an authentic βGlcNAc→3Gal marker in paper chromatography with solvents *A* and *E*; it was well separated from βGlcNAc→6Gal (data not shown). Periodate oxidation and hydrolysis converted the βGlcNAc-→3[^{3}H]Gal-like disaccharide into D-[^{3}H]lyxose (32% yield); [^{3}H]galactose or [^{3}H]threose were not formed (data not shown).

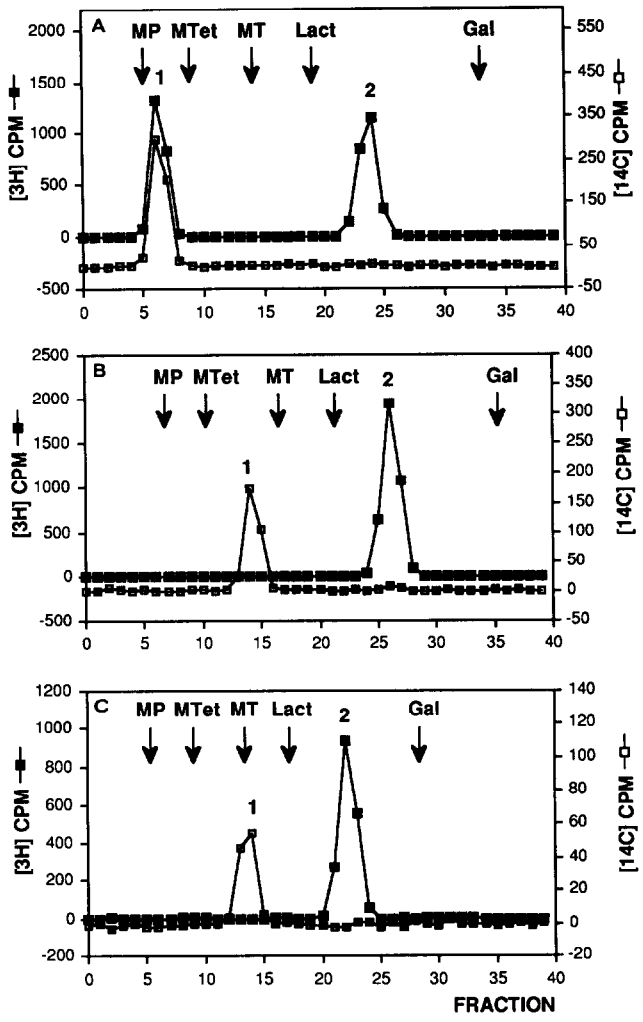


Fig. 4. Paper chromatograms of *B. fragilis* endo-β-D-galactosidase digest: (A) Digest of Peak "hepta" of Fig. 1B. (B) Digest of Peak "octa" of Fig. 1B. (C) Digest of the heptasaccharide, βGlcNAc→3β[6-^{3}H]Gal→4βGlcNAc→3(βGlcNAc→3β[6-^{3}H]Gal→4βGlcNAc→6)[U-^{14}C]Gal ([^{3}H]-**14**). The desalted products were irrigated with solvent *A* for 66, 63, and 46 h, respectively, and the dried chromatograms were counted for radioactivity. (A): Peak 1, pentasaccharides, βGlcNAc→3(β[6-^{3}H]Gal→4βGlcNAc→6)β[U-^{14}C]Gal→4GlcNAc ([^{3}H]-**7**) and β[6-^{3}H]Gal→4βGlcNAc→3(βGlcNAc→6)β[U-^{14}C]Gal→4GlcNAc ([^{3}H]-**8**). Peak 2, βGlcNAc→3[6-^{3}H]Gal. (B): Peak 1, tetrasaccharide, βGlcNAc→3(βGlcNAc→6)β[U-^{14}C]Gal→4GlcNAc. Peak 2, βGlcNAc→3[6-^{3}H]Gal. (C): Peak 1, trisaccharide, βGlcNAc→3(βGlcNAc→6)[U-^{14}C]Gal. Peak 2, βGlcNAc→3[6-^{3}H]Gal. Markers as in legend to Fig. 2.

This proved the presence of a (1→3) linkage, and the absence of (1→2) and (1→4) linkages in the disaccharide, establishing its most likely structure as βGlcNAc→3[6-^{3}H]Gal.

The slower moving peak of Fig. 4A (Peak 1) migrated between maltopentaose and maltotetraose markers; it proved to be an equimolar mixture of the pentasaccharides, βGlcNAc→3(β[6-^{3}H]Gal→4βGlcNAc→6)β[U-^{14}C]Gal→4GlcNAc ([^{3}H]-7) and β[6-^{3}H]Gal→4βGlcNAc→3(βGlcNAc→6)β[U-^{14}C]Gal→4GlcNAc ([^{3}H]-**8**) (see below).

*Characterization of the pentasaccharide fraction released from the heptasaccharides [^{3}H]-**2** and [^{3}H]-**3** by endo-β-D-galactosidase.* — The pentasaccharide fraction from Fig. 4A (Peak 1) contained 51.5% of the total ^{3}H and all the ^{14}C radioactivity present in the endo-β-D-galactosidase digest. It migrated, in paper chromatography, at the same rate as the pentasaccharide mixture obtained by partial degalactosylation of hexasaccharide, βGal→4βGlcNAc→3(βGal→4βGlcNAc→6)β[U-^{14}C]Gal→4GlcNAc, with *Escherichia coli* β-D-galactosidase[6] (see Table I). These findings suggested that the pentasaccharide fraction of Fig. 4A contained a mixture of βGlcNAc→3(β[6-^{3}H]Gal→4βGlcNAc→6)β[U-^{14}C]Gal→4GlcNAc ([^{3}H]-**7**) and β[6-^{3}H]Gal→4βGlcNAc→3(βGlcNAc→6)β[U-^{14}C]Gal→4GlcNAc ([^{3}H]-**8**).

This conclusion was confirmed by digesting the pentasaccharide mixture with jack bean *N*-acetyl-β-D-hexosaminidase. Paper chromatography of the digest revealed equimolar amounts of the isomeric tetrasaccharides, β[6-^{3}H]Gal→4βGlcNAc→6β[U-^{14}C]Gal→4GlcNAc ([^{3}H]-**9**) and β[6-^{3}H]Gal→4βGlcNAc→3β[U-^{14}C]Gal→4GlcNAc ([^{3}H]-**10**) (Fig. 3B and Table I). The two tetrasaccharides were further characterized by WGA-agarose chromatography (data not shown). The slowly migrating tetrasaccharide (Peak 1) was retained relatively strongly by the lectin (Δ 16.4), in a manner very characteristic for tetrasaccharide, βGal→4βGlcNAc→6βGal→4GlcNAc[11]. In contrast, the faster moving tetrasaccharide (Peak 2), which does not contain any βGlcNAc→6Gal sequences, was not appreciably retained by WGA-agarose (Δ 1.6). These findings implied that the pentasaccharide fraction was a 1:1 mixture of oligosaccharides [^{3}H]-**7** and [^{3}H]-**8**, confirming that the original heptasaccharide was an equimolar mixture of [^{3}H]-**2** and [^{3}H]-**3**.

*Analysis of the heptasaccharides [^{14}C]-**2** and [^{14}C]-**3** that were formed early during the* N-*acetylglucosaminylation reaction.* — The heptasaccharide fraction from Fig. 1A, containing the oligosaccharides [^{14}C]-**2** and [^{14}C]-**3**, represented a middle stage in the *N*-acetylglucosaminylation reaction, and contained molecules formed relatively early in the hexa-to-hepta transformation. Thus, its composition reflects largely the branch specificity of the first *N*-acetylglucosaminylation reaction. This heptasaccharide fraction was analyzed by successive treatments with endo-β-D-galactosidase and *N*-acetyl-β-D-hexosaminidase. The result was a mixture of tetrasaccharides that could be separated by paper chromatography into equimolar amounts of [^{14}C]-**9** and [^{14}C]-**10** (data not shown). Thus, even the heptasaccharide fraction from Fig. 1A was a 1:1 mixture of the heptasaccharides [^{14}C]-**2** and [^{14}C]-**3**.

*Characterization of the octasaccharide fraction formed in the (1→3)-*N*-acetyl-β-*

D-glucosaminyltransferase reaction of the hexasaccharide **1**. — Octasaccharide [^{3}H]-**4** migrated, in paper chromatography, slower than the maltoheptaose marker (see Table I). Its digestion with jack bean *N*-acetyl-β-D-hexosaminidase resulted in complete cleavage releasing the hexasaccharide [^{3}H]-**1** (data not shown). Treatment of the octasaccharide fraction from Fig. 1B with endo-β-D-galactosidase from *B. fragilis* gave two components (Fig. 4B). Peak 1 contained all ^{14}C label and migrated like the authentic tetrasaccharide marker, βGlcNAc→3(βGlcNAc→6)βGal→4GlcNAc[3]. Peak 2 contained all ^{3}H radioactivity and migrated like the disaccharide marker βGlcNAc→3Gal[4]. These results, combined with the structural analysis of the heptasaccharide fraction, indicated that the structure of the octasaccharide formed in the *N*-acetylglucosaminylation reaction of Fig. 1B is βGlcNAc→3β[6-^{3}H]Gal→4βGlcNAc→3(βGlcNAc→3β[6-^{3}H]Gal→4βGlcNAc→6)β[U-^{14}C]Gal→4GlcNAc.

Enzymic in vitro *synthesis and characterization of the pentasaccharide βGal→4βGlcNAc→3(βGal→4βGlcNAc→6)[U-^{14}C]Gal ([^{14}C-***11***])*. — The pentasaccharide [^{14}C]-**11** was synthesized by incubating βGlcNAc→3(βGlcNAc→6)[U-^{14}C]Gal with (1→4)-β-D-galactosyltransferase and UDP-D-galactose under conditions that exhausted the acceptor completely. Paper chromatography of the product (Table I), of a partially galactosylated primer, βGlcNAc→3(βGal→4βGlcNAc→6)[U-^{14}C]Gal (**17**)[6], and of the trisaccharide primer[3], confirmed that two D-galactosyl groups were transferred to each primer molecule.

*Incubation of the pentasaccharide [^{14}C]-***11** *and UDP-GlcNAc with human serum; isolation of hexasaccharide fractions from early, middle, and late reaction mixtures.* — Incubation of [^{14}C]-**11** and UDP-GlcNAc with human serum for short, medium, and prolonged periods gave reaction mixtures that represented 16.5, 58, and 88% glycosylation of available sites, respectively. They all were separated into three components by paper chromatography (Fig. 5). The three peaks represented the primer pentasaccharide **11** ("penta"), the mixed hexasaccharides **12** and **13** ("hexa"), and the heptasaccharide **14** ("hepta") (see Chart 1 for the systematic and abbreviated structures). The mixtures containing the hexasaccharides **12** and **13** were isolated from the three reactions, and the compositions were established by cleaving them with endo-β-D-galactosidase into mixtures of tetrasaccharides **17** and **18** (Fig. 6). The tetrasaccharide mixtures were then digested with *N*-acetyl-β-D-hexosaminidase which converted them into mixtures of trisaccharides **19** and **20**. The latter compounds were separated by paper chromatography (Fig. 7), and the results showed that the 1:1 mixtures of **19** and **20** were derived from the mixtures of hexasaccharides **12** and **13** at all stages of the *N*-acetylglucosaminylation reaction of pentasaccharide **11**. Accordingly, this reaction appears to proceed in a random manner, without noticeable branch-specificity, in the transformation from the pentasaccharide to hexasaccharides **12** and **13**, and also without any selectivity in the transformation of hexasaccharides **12** and **13** into heptasaccharide **14**.

*Characterization of the heptasaccharide fraction formed in the (1→3)-*N*-acetyl-β-D-glucosaminyltransferase reaction of pentasaccharide* **11**. — The heptasaccharide peak of Figs. 5A, B, and C migrated slower than the maltoheptaose marker (see Table I).

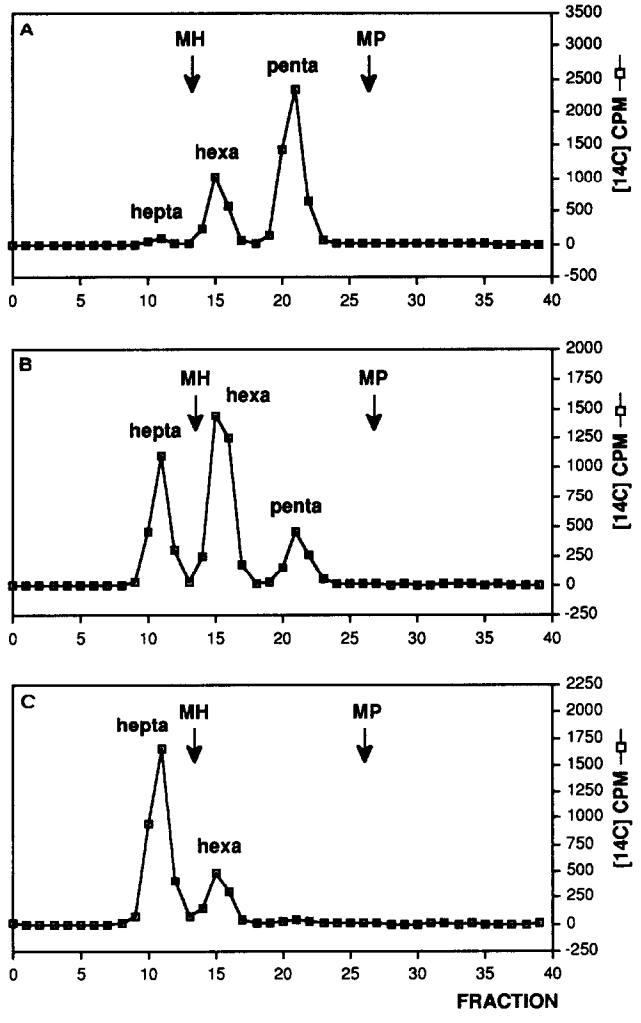


Fig. 5. Paper chromatographic separation of the products from the (1→3)-*N*-acetyl-*β*-D-glucosaminyl-transferase reactions of *β*Gal→4*β*GlcNAc→3(*β*Gal→4*β*GlcNAc→6)[U-^{14}C]Gal ([^{14}C]-**11**). Increasing incubation times were used so that 16.5, 58, and 88% of the available acceptor sites had reacted in the experiments of panels A, B, and C, respectively. The desalted products were irrigated with solvent *A* for 9 d, and the dried chromatograms were counted for radioactivity. The peaks labeled "hepta" represent the heptasaccharide, *β*GlcNAc→3*β*Gal→4*β*GlcNAc→3(*β*GlcNAc→3*β*Gal→4*β*GlcNAc→6)[U-^{14}C]Gal ([^{14}C]-**14**). The peaks labeled "hexa" represent mixtures of the hexasaccharides, *β*GlcNAc→3*β*Gal→4*β*GlcNAc→3(*β*Gal→4*β*GlcNAc→6)[U-^{14}C]Gal ([^{14}C]-**12**) and *β*Gal→4*β*GlcNAc→3(*β*GlcNAc→3*β*Gal→4*β*GlcNAc→6)[U-^{14}C]Gal ([^{14}C]-**13**). The peaks labeled "penta" represent the substrate pentasaccharide. The arrows marked MH and MP show the positions of maltoheptaose and maltopentaose, respectively.

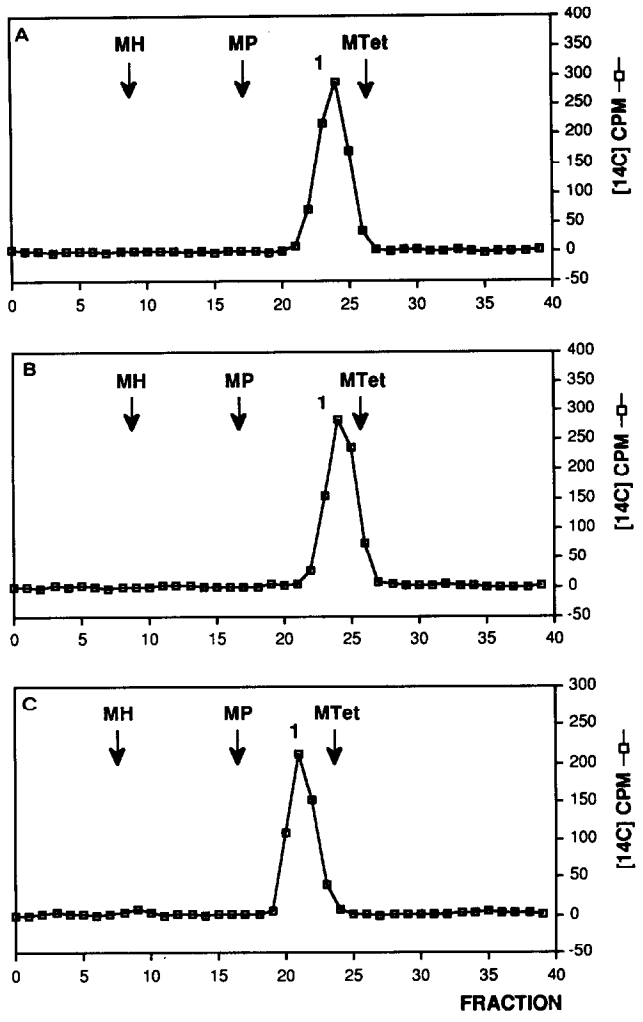


Fig. 6. Paper chromatograms of *B. fragilis* endo-β-D-galactosidase digests: (A) Digest of Peak "hexa" of Fig. 5 A. (B) Digest of Peak "hexa" of Fig. 5 B. (C) Digest of Peak "hexa" of Fig. 5 C. The desalted products were irrigated with solvent *A* for 5 d, and the dried chromatograms were counted for radioactivity. Peak 1, mixture of tetrasaccharides, βGlcNAc→3(βGal→4βGlcNAc→6)[U-^{14}C]Gal ([^{14}C]-**17**) and βGal→4βGlcNAc→3(βGlcNAc→6)[U-^{14}C]Gal ([^{14}C]-**18**). The arrows marked MH, MP, and MTet show the positions of maltopheptaose, maltopentaose, and maltotetraose, respectively.

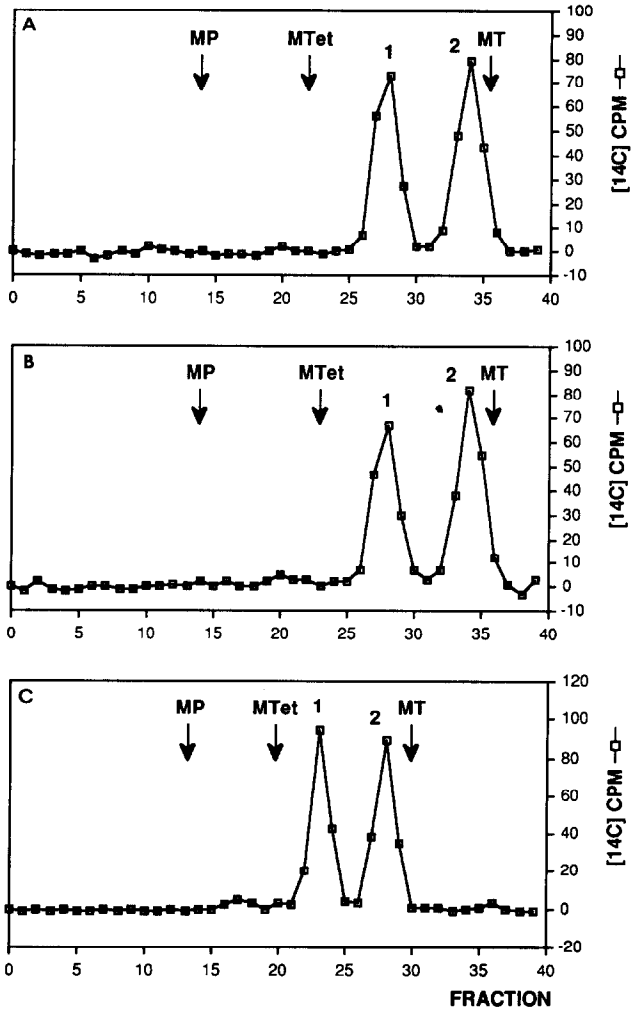


Fig. 7. Paper chromatograms of jack bean *N*-acetyl-β-D-hexosaminidase digests. (A) Digest of Peak 1 of Fig. 6 A. (B) Digest of Peak 1 of Fig. 6 B. (C) Digest of Peak 1 of Fig. 6 C. The desalted products were irrigated with solvent *A* for 5 d, and the dried chromatograms were counted for radioactivity. Peak 1, βGal→4βGlcNAc→6[U-^{14}C]Gal ([^{14}C]-**19**). Peak 2, βGal→4βGlcNAc→3[U-^{14}C]Gal ([^{14}C]-**20**). Markers as in the legend to Fig. 3.

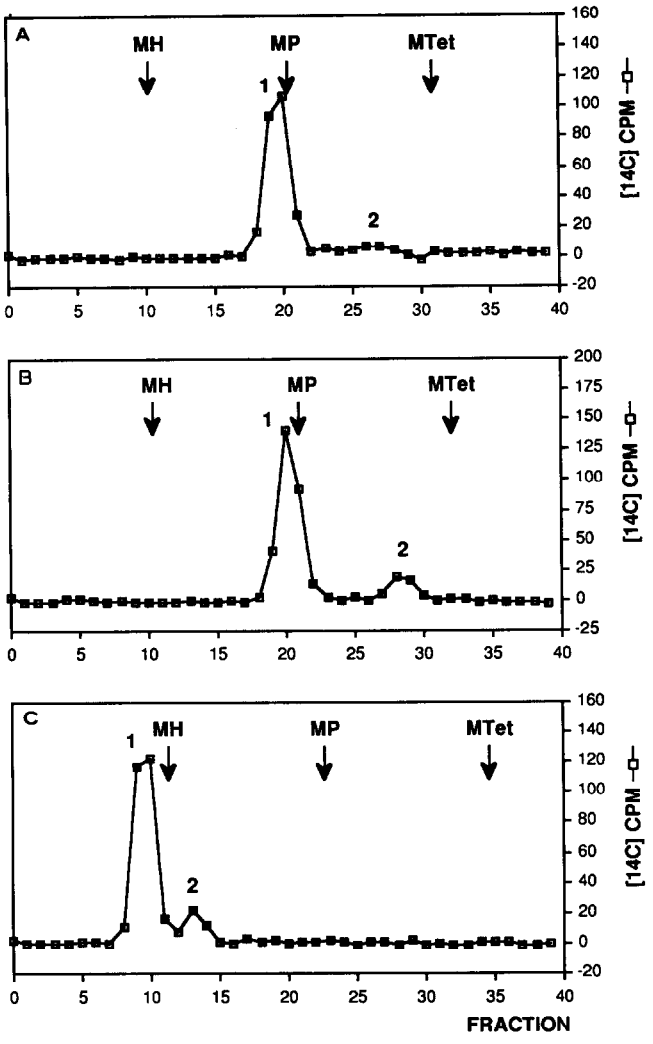


Fig. 8. Paper chromatographic separation of the products from the (1→3)-*N*-acetyl-β-D-glucosaminyltransferase reactions: (A) Products from βGlcNAc→3(βGal→4βGlcNAc→6)[U-^{14}C]Gal ([^{14}C]-**17**). (B) Products from βGal→ 4βGlcNAc→3(βGlcNAc→6)[U-^{14}C]Gal ([^{14}C]-**18**). (C) Products from βGal→4βGlcNAc→3(βGal→4βGlcNAc→6)[U-^{14}C]Gal ([^{14}C]-**11**). The desalted products were irrigated with solvent *A* for 8 d, and the dried chromatograms were counted for radioactivity. (A): Peak 1, βGlcNAc→3(βGlcNAc→3βGal→4βGlcNAc→6)[U-^{14}C]Gal ([^{14}C]-**15**). Peak 2, the substrate tetrasaccharide **17**. (B): Peak 1, βGlcNAc→3βGal→4βGlcNAc→3(βGlcNAc→6)[U-^{14}C]Gal ([^{14}C]-**16**). Peak 2, the substrate tetrasaccharide, [^{14}C]-**18**. (C): Peak 1, βGlcNAc→3βGal→4βGlcNAc→3(βGlcNAc→3βGal→4βGlcNAc→6)[U-^{14}C]Gal ([^{14}C]-**14**). Peak 2, hexasaccharides, βGlcNAc→3βGal→4βGlcNAc→3(βGal→4βGlcNAc→6)[U-^{14}C]Gal ([^{14}C]-**12**) and βGal→4βGlcNAc→3(βGlcNAc→3βGal→4βGlcNAc→6)[U-^{14}C]Gal ([^{14}C]-(**13**). Markers as in the legend to Fig. 6.

Digestion of the heptasaccharide fraction from Fig. 5B with *N*-acetyl-β-D-hexosaminidase gave a complete cleavage releasing pentasaccharide **11** (data not shown). The heptasaccharide [^{3}H]-**14**, that was formed in the incubation of pentasaccharide, β[6-^{3}H]Gal→4βGlcNAc→3(β[6-^{3}H]Gal→4βGlcNAc→6)[U-^{14}C]Gal ([^{3}H]-**11**) with UDP-GlcNAc and serum, was cleaved completely by endo-β-D-galactosidase. The hydrolysis products were identified by paper chromatography as βGlcNAc→3[6-^{3}H]Gal and βGlcNAc→3(βGlcNAc→6)[U-^{14}C]Gal (Fig. 4C). The disaccharide that behaved on chromatography like βGlcNAc→3[6-^{3}H]Gal was oxidized with periodate at pH 3.6, and subsequently cleaved with acid to yield [6-^{3}H]lyxose (data not shown). These results established that the structure of the heptasaccharide formed from pentasaccharide [^{3}H]-**11** was βGlcNAc→3β[6-^{3}H]Gal→4βGlcNAc→3(βGlcNAc→3β[6-^{3}H]Gal→4βGlcNAc→6)[U-^{14}C]Gal ([^{3}H]-**14**).

(1→3)-N-*Acetyl-β-D-glucosaminyltransferase reactions using the branched tetrasaccharides* **17** *and* **18**, *as well as the branched pentasaccharide* **11** *as acceptors.* — The incubation mixtures obtained in parallel experiments from [^{14}C]-**17**, [^{14}C]-**18**, and [^{14}C]-**11** with UDP-GlcNAc and serum gave the paper chromatograms shown in Fig. 8. All three acceptors reacted in a nearly quantitative manner, yielding oligosaccharides that migrated more slowly than the original substrates. As judged from the mobilities of the products, **11** had been converted mainly into **14**, whereas the products of **17** and **18** contained one newly acquired GlcNAc unit each, representing most likely [^{14}C]-**15** and [^{14}C]-**16**, respectively. The nearly complete reaction of tetrasaccharide **18** is noteworthy, because Piller and Catron[1] reported that it is a poor acceptor in the reaction catalyzed by serum (1→3)-*N*-acetyl-β-D-glucosaminyltransferase.

DISCUSSION

The presently reported experiments led to the *in vitro* biosynthesis and structural characterization of twenty radiolabeled oligo-(*N*-acetyllactosamino)glycans shown in Chart I and Table I. These oligosaccharides are of importance as primers for further biosynthetic studies in our laboratory; they are valuable also as chromatographic markers in the structural analysis of poly-(*N*-acetyllactosamino)glycans from teratocarcinoma cells[15–18].

Most of the newly synthesized saccharides were obtained as mixtures of two isomeric; biantennary molecules. Generally, these mixtures could not be resolved by chromatographic methods*. However, the individual components of each mixture could be detected, quantitatively determined, and analyzed structurally by use of enzymic cleavage reactions that transformed them in a few steps into a mixture of βGal→4βGlcNAc→6βGal→4GlcNAc (**9**) and βGal→4βGlcNAc→3βGal→4GlcNAc (**10**), or into a mixture of βGal→4βGlcNAc→6Gal (**19**) and βGal→4βGlcNAc→3βGal

* Recently, pentasaccharides **7** and **8** have been separated partially by prolonged paper chromatography in Solvent *A*[19].

(**20**) (see Schemes 1 and 2). These two linear tetrasaccharides, or the two linear trisaccharides, could then be separated from each other by paper chromatography[11], and by WGA-agarose chromatography[11,*]. A similar method has been applied recently[6] to analyze the composition of mixtures containing oligosaccharides **7** and **8**, or **17** and **18**.

In summary, our results indicate that both branches of hexasaccharide **1** and pentasaccharide **11** are elongated by the serum (1→3)-*N*-acetyl-β-D-glucosaminyltransferase. Furthermore, it appears that both steps in the two-step reactions catalyzed by the serum (1→3)-*N*-acetyl-β-D-glucosaminyltransferase proceeded without appreciable branch specificity. This conclusion implies that partial *N*-acetyl-β-D-glucosaminyltransferase reactions may be used to biosynthesize well defined oligosaccharide mixtures or "oligosaccharide libraries".

When tested with the partially galactosylated acceptors **17** and **18**, the serum enzyme revealed, in our hands, an acceptor specificity similar to that reported for the analogous enzyme from Novikoff tumor cell ascites fluid[2]; it catalyzed as easily the near complete elongation of β-(1→6) and β-(1→3) branches of tetrasaccharides **17** and **18**, respectively, as well as the elongation of both branches of pentasaccharide **11**. It is interesting that **18** appeared to react a little more slowly than **17** with the serum enzyme (see Fig. 8). Piller and Cartron[1] have also reported that **18** is a poor acceptor as compared to **11**.

The present results were obtained by use of a soluble transferase and free oligosaccharides as its substrates. However, the enzyme reaction *in vivo* is carried out by a membrane-bound enzyme, which may impose a change to the flexibility of the catalytic site. Moreover, the actual acceptors *in vivo* are linked to one of the side-chains in N- or O-glycans, or ceramides, which may influence the enzyme accessibility. Despite these uncertainties, the present results are of interest also in the context of the biosynthesis of poly-(*N*-acetyllactosamino)glycans *in vivo*. A prevalent "backbone structure" among many branched poly-(*N*-acetyllactosamino)glycans is that of a long linear chain of β-(1→3)-linked *N*-acetyllactosamine units, to which short (βGal→4βGlcNAc)-linked branches are attached at O-6 of some of the galactose units[17,20–25]. The branch specificities of the (1→3)-*N*-acetyl-β-D-glucosaminyltransferases of serum and of Novikoff tumor cell ascites fluid[2] do not correspond to these structures. Therefore, it is possible that other factors than the "branch specifities" of the (1→3)-*N*-acetyl-β-D-glucosaminyltransferases determine the pathways of the *in vivo* elongation of oligo-(*N*-acetyllactosamino)glycans. One possibility is that the (1→3)-β-D-GlcNAc- and (1→6)-β-D-GlcNAc-transferases may be spatially separated in the synthesizing cells, the latter enzyme acting later during the growth of the oligosaccharide chains. Recently, Levery *et al.*[26] and Bruntz *et al.*[27] have isolated backbone oligosaccharides, that are elongated at the (1-6)- as well as at the (1→3)-branches, reflecting the substrate specificities of the known (1→3)-*N*-acetyl-β-D-glucosaminyltransferases.

* In contrast to tetrasaccharide **9** and trisaccharide **19**, tetrasaccharide **10** and trisaccharide **20** are retarded only very little in the WGA-agarose column[19].

It is noteworthy that partial α-D-galactosylation of the biantennary hexasaccharide **1**, catalyzed by calf thymus (1→3)-α-D-galactosyltransferase, gave in a preliminary experiment the heptasaccharide, βGal→4βGlcNAc→3(αGal→3βGal→4βGlcNAc→6)-βGal→4GlcNAc in a rather pure form[28]. This implies that the two OH-3 in the two nonreducing D-galactosyl groups of the biantennary hexasaccharide **1** possess quite different relative reactivities for the (1→3)-α-D-galactosyltransferase and the (1→3)-*N*-acetyl-β-D-glucosaminyltransferase reactions.

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