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N-Acetyllactosaminooligosaccharides that contain the β-D-Glc*p*NAc-(1→6)-D-Gal or β-D-Glc*p*NAc-(1→6)-D-GalNAc sequences reveal reduction-sensitive affinities for wheat germ agglutinin*,†

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ABSTRACT

Affinity chromatography of unreduced oligosaccharides on a small column of immobilized wheat germ agglutinin (WGA) revealed high-binding affinities for several radiolabeled molecules containing at the reducing end either β-D-Glc*p*NAc-(1→6)-D-Gal, β-D-Glc*p*NAc-(1→6)-β-D-Gal*p*-(1→4)-D-GlcNAc, β-D-Glc*p*NAc-(1→6)-β-D-Gal*p*-(1→4)DGlc, D-Glc*p*NAc-(1→3)-[β-D-Glc*p*NAc-(1→6)]-D-Gal, β-D-Glc*p*NAc-(1→6)-D-GalNAc, or β-D-Gal*p*-(1→3)-[β-D-Glc*p*NAc-(1→6)]-D-GalNAc sequences. Reduction changed the binding affinities remarkably: The sequences carrying a D-galactose or 2-acetamido-2-deoxy-D-galactose residue at the reducing end lost most of their affinities, but the sequences containing a D-glucose or 2-acetamido-2-deoxy-D-glucose residue at the reducing end gained additional affinity upon reduction. These findings emphasize the role of the unreduced, 6-O-substituted D-galactose and 2-acetamido-2-deoxy-D-galactose residues for the binding of saccharides to WGA, which has been recognized previously as a lectin specific for oligosaccharides containing a 2-acetamido-2-deoxy-D-glucose or sialic acid unit. The results suggested also that WGA–agarose chromatography of alditols may become a valuable method for the fractionation of oligo-*N*-acetyllactosaminoglycans and related saccharides.

INTRODUCTION

Previous work has shown that oligosaccharides βGlcNAc→6Gal (**1a**), βGlcNAc→6βGal→4GlcNAc (**7a**), βGal→4βGlcNAc→6βGal→4GlcNAc (**8a**), βGlcNAc→3(β-GlcNAc→6)Gal (**5a**), and βGlcNAc→3(βGlcNAc→6)βGal→4GlcNAc (**9a**)*** bind to agarose-linked wheat germ agglutinin (WGA)[1–4]. In these early experiments, we observed that the oligosaccharides carrying a D-galactose residue at the reducing end give two peaks in WGA-agarose chromatography[2–4]. The two peaks were assumed to represent mutarotational isomers, because both of them gave again the original double peak profiles upon rechromatography[2,4]. As an attempt to improve their separations in

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*** See Table I for abbreviated structures.

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WGA–agarose chromatography we studied the *N*-acetyllactosaminooligosaccharides in reduced form.

EXPERIMENTAL

Paper chromatography. — Chromatography of desalted oligosaccharides on Whatman No. 3 paper was carried out with the upper phase 4:1:5 (v/v) butanol–acetic acid–water (Solvent A) or with 10:1:2 (v/v) butanol–ethanol–water (Solvent B). The running times varied from 16 to 48 h for monosaccharides and up to eight days for penta- and hexa-saccharides. The radioactivity in paper chromatograms was analyzed as previously described[3]. Unlabeled alditols were stained by a silver nitrate spray in acetone followed by a sodium hydroxide spray. The marker trisaccharide, β-D-Gal*p* (1→4)-β-D-Glc*p*NAc-(1→3)-D-Gal, was described in ref. 3, and the marker disaccharide β-D-Glc*p*NAc-(1→3)-D-Gal in ref. 1. Other markers were: M_3, maltotriose; M_4, maltotetraose; M_5, maltopentaose; and M_7 maltoheptaose.

Preparation of the reducing oligosaccharides. (**1a**–**14a**). — The radiolabeled compounds **1a**[1], **2a**[3], **5a**[4], **7a**[4], **8a**[3], and **9a**[4] (see Table I for structures and numbering) have been described previously.

Pentasaccharide βGal→4βGlcNAc→3(βGal→4βGlcNAc→6)[^{14}C]Gal(^{14}C-**6a**) was obtained by galactosylation of βGlcNAc→3(βGlcNAc→6)[^{14}C]Gal(^{14}C-**5a**) with UDP-Gal (50 nmol) and *N*-acetyllactosamine synthase[5] (EC 2.4.1.90) (50 mU) from bovine milk in a mixture that contained, in 100 μL, Tris·HCl, pH 7.5, (5 μmol) and $MnCl_2$ (1 μmol). The pentasaccharide ^{14}C-**6a** was isolated by paper chromatography with Solvent A (R_{M5} 0.84;R_{M7} 1.71). It was cleaved completely by a treatment with *Diplococcus pneumoniae* β-D-galactosidase[6] (Boehringer, Mannheim, FRG) (EC 3.2.1.23), which proved[7] that it contained distal (1→4)-linked β-D-galactosyl groups. Partial cleavage experiments with *Escherichia coli* β-D-galactosidase (Sigma Chemical Co., St. Louis, MO, U.S.A.) showed that it contained two distal D-galactose units[8].

Tetrasaccharide βGlcNAc→3β[U-^{14}C]Gal→4βGlcNAc→6Gal (^{14}C-**3a**) was prepared by incubating trisaccharide β[U-^{14}C]Gal→4βGlcNAc→6Gal (^{14}C-**2a**) with UDP-GlcNAc and human serum as described[4]. The serum contains β-(1→3)-*N*-acetyl-D-glucosaminyltransferase activity (EC 2.4.1.149) that catalyzed the formation of **3a** (R_{M3} 0.57, R_{M4} 0.88 in Solvent A) in a yield of 11%. Tetrasaccharide (^{14}C-**3a**) was characterized by cleavage with endo-β-D-galactosidase[3] from *Escherichia freundii* (Seikagaku, Tokyo, Japan) into a radiolabeled disaccharide that was identified as βGlcNAc→3[U-^{14}C]Gal by paper chromatography in Solvent A (R_{GAL} 0.71; R_{LACT} 1.21). The disaccharide was further cleaved by β-*N*-acetyl-D-hexosaminidase into radiolabeled galactose that was identified by paper chromatography in Solvent A. Periodate oxidation and subsequent acid hydrolysis[4], according to Hough[9], of the disaccharide gave [^{14}C]lyxose (31% yield) that was identified by paper chromatography in Solvent A. These results established that the newly introduced 2-acetamido-2-deoxy-β-D-glucosyl group was linked to the trisaccharide acceptor at O-3.

The linear pentasaccharide βGal→4βGlcNAc→3β[U-^{14}C]Gal→4βGlcNAc→6

Gal (^{14}C-**4a**) was obtained by enzymic (1→4)-β-D-galactosylation of tetrasaccharide **3a** (R_{M5} 0.77, R_{M7} 1.64 in solvent A). Its structure was characterized by cleavage with *D. pneumoniae* β-D-galactosidase that released the linear tetrasaccharide **3a**, thus establishing that the newly introduced β-D-galactosyl group was linked to the acceptor at O-4. In a confirmatory test, endo-β-D-galactosidase (from *E. freundii*) cleaved ^{14}C-**4a** into the radiolabeled trisaccharide βGal→4βGlcNAc→3[U-^{14}C]Gal, which was identified by paper chromatography in solvent A (R_{LAC} 0.69; R_{M3} 0.96) (see ref. 3), and by further sequencing.

The branched hexasaccharide βGal→4βGlcNAc→3(βGal→4βGlcNAc→6)β-[^{14}C]Gal→4GlcNAc (^{14}C-**10a**) was prepared by galactosylating βGlcNAc→3(βGlcNAc→6)β[^{14}C]Gal→4)GlcNAc as described above. The hexasaccharide was isolated by paper chromatography in Solvent A (R_{M5} 0.72, R_{M7} 1.49) (see ref. 8). It, too, was cleaved completely by treatment with *D. pneumoniae* β-D-galactosidase. Partial cleavage experiments with *E. coli* β-D-galactosidase showed that it, too, contained two distal D-galactose units[8].

The linear tetrasaccharide β[U-^{14}C]Gal→4βGlcNAc→6βGal→4Glc (^{14}C-**11a**) was prepared by enzymic (1→4)-β-D-[^{14}C]galactosylation of βGlcNAc→6βGal→4Glc; the trisaccharide acceptor was purchased from Sigma.

The trisaccharide βGal→3(β[^{14}C]GlcNAc→6)GalNAc (^{14}C-**13a**) was synthesized by incubating UDP-[^{14}C]GlcNAc (Amersham Corp.) and βGal→3GalNAc (**14a**) (Bio-Carb Chemicals AB, Lund, Sweden) with crude (1→6)-*N*-acetyl-β-D-glucosaminyltransferase (EC 2.4.1.148) of hog gastric mucosa[10] essentially as described by Seppo *et al.*[4] The tritiated disaccharide acceptor, βGal→3[^{3}H]GalNAc (^{3}H-**14a**) was prepared from βGal→3GalNAc (**14**) by a catalytic ^{3}H exchange process (TR 7) in Amersham; the product was filtered through Dowex 1 (AcO^-) and Dowex 50 (H^+) ion-exchange resins, and purified by paper chromatography. Trisaccharide ^{3}H-**13a**, βGal→3(βGlcNAc→6)[^{3}H]GalNAc, was prepared by incubating βGal→3[^{3}H]GalNAc with UDP-GlcNAc and the crude (1→6)-*N*-acetyl-β-D-glucosaminyltransferase as described above. The radiolabeled trisaccharide **13a** preparations were purified by paper chromatography in Solvent A (R_{GAL} 0.83, R_{LAC} 1.41), and subsequently in Solvent B (R_{GAL} 0.43, R_{LAC} 1.54).

Structural characterization of ^{3}H-**13a** was carried out by reducing it with $NaBH_4$ (see below) to give βGal→3(βGlcNAc→6)[^{3}H]GalNAcol (^{3}H-**13b**), which was isolated by paper chromatography. Prolonged treatment with *E. coli* β-D-galactosidase (EC 3.2.1.23) (Sigma) gave βGlcNAc→6[^{3}H]GalNAcol (^{3}H-**12b**). This was cleaved further by jack bean *N*-acetyl-β-D-hexosaminidase (EC 3.2.1.52) into [^{3}H]GalNAcol that was identified by paper chromatography in borate solution[11]. The disaccharide alditol could also be cleaved into *N*-acetyl[^{3}H]serinol by a procedure[12] involving periodate oxidation, subsequent $NaBH_4$ reduction, acid hydrolysis, and *N*-reacetylation. The procedure was carried out as described previously[13], and *N*-acetyl[^{3}H]serinol was identified by paper chromatography, which separated it clearly from radiolabeled 2-acetamido-2-deoxythreitol, 2-acetamido-2-deoxyarabinitol, and 2-acetamido-2-deoxygalactitol. The formation of *N*-acetyl[^{3}H]serinol proved that the [^{3}H]GalNAc unit was substituted at O-6 in the original trisaccharide ^{3}H-**13a**. ^{14}C-labeled trisaccharide **13a**, βGal→

3(β[^{14}C]GlcNAc→6)GalNAc, in turn, was cleaved by *N*-acetyl-β-D-hexosaminidase into [^{14}C]GlcNAc.

The disaccharide β[U-^{14}C]GlcNAc→6GalNAc (^{14}C-**12a**) was prepared by repeated and prolonged β-D-galactosidase (*E. coli*) digestions of trisaccharide ^{14}C-**13a**.

Preparation of alditols **1b–15b**. — Oligosaccharides **1a–14a**, and *N,N'*-diacetylchitobiose (**15a**) (Sigma), were reduced with $NaBH_4$ essentially as described by Rasilo and Renkonen[11]. *N,N'*-Diacetylchitobiose (**15a**) was reduced also with $NaBT_4$. The alditols were isolated by passing the borate-free reaction mixtures through small columns of Dowex 1 (OH^-) anion-exchange resin[14], which retain unreduced saccharides much more than alditols. Subsequent paper chromatography gave pure alditols that were recovered by water extraction and lyophilization.

The branched pentasaccharide alditol βGal→4βGlcNAc→3(βGal→4βGlcNAc →6)[^{14}C]Galol (^{14}C-**6b**) was characterized by complete hydrolysis with acid. Paper chromatography with Solvent A revealed that the digest contained pure [^{14}C]Galol, and was free of [^{14}C]Gal. The trisaccharide alditol βGlcNAc→3(βGlcNAc→6)[^{14}C]Galol (^{14}C-**5b**) was characterized in an analogous manner by cleavage with jack bean *N*-acetyl-β-D-hexosaminidase (EC 3.2.1.52). Complete acid hydrolysis of the branched tetrasaccharide alditol, βGlcNAc→3(βGlcNAc→6)βGal→4[^{14}C]GlcNAcol (^{14}C-**9b**), followed by *N*-reacetylation gave [^{14}C]GlcNAcol that was identified by ordinary paper chromatography (Solvent A), and subsequently by borate paper chromatography[11]. Characterization of the trisaccharide alditol βGal→3(βGlcNAc→6)[^{3}H]GalNAcol (^{3}H-**13b**) has been described above (see under the reducing saccharides). The disaccharide alditol βGlcNAc→4[1-^{3}H]GlcNAcol (^{3}H-**15b**) moved in chromatography on paper with Solvent A like the unlabeled alditol βGlcNAc→4GlcNAcol (R_{GAL} 1.00). Our standard treatment[1] with *N*-acetyl-β-D-hexosaminidase cleaved 68% of ^{3}H-**15b** and released [1-^{3}H]GlcNAcol that was identified by paper chromatography in borate solution[11].

WGA–agarose chromatography. — Chromatography on agarose-bound WGA (1.65 mg WGA/mL of 4% beaded agarose; Pharmacia, Uppsala, Sweden) was carried out at +6–7° in a 0.7 × 12 cm column[1]. (The same column has been in continuous use for 26 months, and its height has decreased with time from 14 to 8 cm). The column was equilibrated and irrigated with 10mM phosphate buffer (pH 7.1) containing 0.15M NaCl and 0.02% NaN_3, at a flow rate of 0.1 mL/min. Fractions (0.55 mL) were collected and assayed for radioactivity. The elution was carried out by collecting fractions 1–40 with the equilibration buffer, after which 0.2M 2-acetamido-2-deoxy-D-glucose in the equilibration buffer was applied to the column; fractions 41–70 were collected with this eluent, which caused desorption of strongly bound saccharides. For regeneration after a completed experiment, the column head was carefully rinsed with the equilibration buffer, after which the column was washed with the equilibration buffer overnight; the flow rate was adjusted to 0.05 mL/min during the washing period.

The column was calibrated monthly with radiolabeled galactose or xylose, both of which are assumedly eluted at the void volume. With rapidly eluted [^{14}C]oligosaccharides, [^{3}H]galactose was cochromatographed as an internal void-volume marker; with

TABLE I

List of reducing oligosaccharides (**1a–16a**) and corresponding alditols (**1b–16b**) examined for affinity to wheat germ agglutinin

Systematic structures of reducing oligosaccharides	*Abbreviated structures of oligosaccharides and corresponding alditols*	
β-D-Glc*p*NAc-(1→6)-D-Gal	**1a**	βGlcNAc→6Gal
	1b	βGlcNAc→6Galol
β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc-(1→6)-D-Gal	**2a**	βGal→4βGlcNAc→6Gal
	2b	βGal→4βGlcNAc→6Galol
β-D-Glc*p*NAc-(1→3)-β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc-(1→6)-D-Gal	**3a**	βGlcNAc→3βGal→4βGlcNAc→6Gal
	3b	βGlcNAc→3βGal→4βGlcNAc→6Galol
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)-D-Gal	**4a**	βGal→4βGlcNAc→3βGal→4βGlcNAc→6Gal
	4b	βGal↓4βGlcNAc→3βGal→4βGlcNAc→6Galol
β-D-Glc*p*NAc 1 ↓ 6 β-D-Glc*p*NAc-(1→3)-D-Gal	**5a**	βGlcNAc→3(βGlcNAc→6)Gal
	5b	βGlcNAc→3(βGlcNAc→6)Galol
β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc 1 ↓ 6 β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc-(1→3)-D-Gal	**6a**	βGal→4βGlcNAc→3(βGal→4βGlcNAc→6)Gal
	6b	βGal→4βGlcNAc→3(βGal→4βGlcNAc→6)Galol
β-D-Glc*p*NAc-(1→6)-β-D-Gal*p*-(1→4)-D-GlcNAc	**7a**	βGlcNAc→6βGal→4GlcNAc
	7b	βGlcNAc→6βGal→4GlcNAcol
β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc-(1→6)-β-D-Gal*p*-(1→4)-D-GlcNAc	**8a**	βGal→4βGlcNAc→6βGal→4GlcNAc
	8b	βGal→4βGlcNAc→6βGal→4GlcNAcol
β-D-Glc*p*NAc 1 ↓ 6 β-D-Glc*p*NAc-(1→3)-β-D-Gal*p*-(1→4)-D-GlcNAc	**9a**	βGlcNAc→3(βGlcNAc→6)βGal→4GlcNAc
	9b	βGlcNAc→3(βGlcNAc→6)βGal→4GlcNAcol

continued

TABLE I (continued)

Systematic structures of reducing oligosaccharides		*Abbreviated structures of oligosaccharides and corresponding alditols*
β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc 1 ↓ 6 β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc-(1→3)-β-D-Gal*p*-(1→4)-D-GlcNAc	**10a** **10b**	βGal→4βGlcNAc→3(βGal→4βGlcNAc→6)βGal→4GlcNAc βGal→4βGlcNAc→3(βGal→4βGlcNAc→6)βGal→4GlcNAcol
β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc-(1→6)-β-D-Gal*p*-(1→4)-D-Glc	**11a** **11b**	βGal→4βGlcNAc→6βGal→4Glc βGal→4βGlcNAc→6βGal→4Glcol
β-D-Glc*p*NAc-(1→6)-D-GalNAc	**12a** **12b**	βGlcNAc→6GalNAc βGlcNAc→6GalNAcol
β-D-Glc*p*NAc 1 ↓ 6 β-D-Gal*p*-(1→3)-D-GalNAc	**13a** **13b**	βGal→3(βGlcNAc→6)GalNAc βGal→3(βGlcNAc→6)GalNAcol
β-D-Gal*p*-(1→3)-D-GalNAc	**14a** **14b**	βGal→3GalNAc βGal→3GalNAcol
β-D-Glc*p*NAc-(1→4)-D-GlcNAc	**15a** **15b**	βGlcNAc→4GlcNAc[a] βGlcNAc→4GlcNAcol
β-D-Glc*p*NAc-(1→4)-β-D-Glc*p*NAc-(1→4)-D-GlcNAc	**16a** **16b**	βGlcNAc→4GlcNAc→4GlcNAc[a] βGlcNAc→4GlcNAc→4GlcNAcol

[a] Not examined.

rapidly eluted [^{3}H]oligosaccharides, [^{14}C]galactose was used in an analogous manner. Another calibration marker, used three or four times a year, was *N,N',N''*-triacetylchitotriose reduced with NaB^3H_4 (ref. 3); this alditol was eluted slightly behind the void volume of the strong eluent, at fraction 55 from the fresh column, and at fraction 49 from the 26-month-old column.

The void volume of the WGA-agarose column decreased from fraction 12 to fraction 8 during the 26-month period of use. To minimize the effect of this gradual change on the data, the results are presented as a difference, $D = V_e - V_o$, where V_e is the peak position of the oligosaccharide, and V_o is the void volume. Even the D-values decreased slowly the 26-month period. The oligosaccharides were desalted by passage through a double bed of Dowex 1 (AcO^-) and Dowex 50 (H^+) ion-exchange resins prior to WGA chromatography.

RESULTS

The structures of the 16 radiolabeled, *N*-acetyllactosamine-type oligosaccharides and their corresponding alditols, studied in the present investigation, are shown in Table I.

*WGA–agarose chromatography of radiolabeled oligosaccharides (**1a–6a**) that carry a* D-*galactose residue at the reducing end; comparison with the corresponding alditols* (**1b–6b**). — A series of chromatograms in a small column of WGA–agarose are illustrated in Fig. 1. The left-side panels (A–F) show the elution profiles obtained with unreduced oligosaccharides **1a–6a**, and the elution profiles of the corresponding alditols **1b–6b** are shown in the right-side panels (G–L).

βGlcNAc→6Gal (**1a**) was strongly bound to the lectin, giving two peaks[1,2] (Fig. 1A), but its corresponding alditol (**1b**) was very weakly bound, giving a single sharp peak (Fig. 1G). The exact binding affinities of the saccharides are suitably described in terms of the number of chromatographic fractions collected between the void volume and the peak position. This number is referred to as the D-value. For the two peaks of disaccharide **1a**, D1 is equal to 18 and D2 to 40. For the single peak of **1b**, D is equal to 1.3. Trisaccharide βGal→4βGlcNAc→6Gal (**2a**) was also relatively strongly bound, giving two peaks (Fig. 1B; D1 12, D2 21). This confirmed our previous findings with impure preparations[3]. Both peak fractions of **2a** gave again two-peak profiles upon rechromatography on WGA–agarose (data not shown). Comparison with Fig. 1H showed that the alditol **2b** gave a single peak that was weakly bound (D 3.0). It is interesting that the nonreducing D-galactosyl end-group of **2a** lessened the binding affinity, as compared to **1a**; there are examples in our data where the nonreducing D-galactosyl end-groups enhanced the binding affinity remarkably (see below). The behavior of the tetrasaccharide βGlcNAc→3βGal→4βGlcNAc→6Gal (**3a**) (Fig. 1C) resembled that of trisaccharide **2a**. The two peaks of **3a** were considerably retarded (D1 14, D2 23), and both peak fractions gave again two-peak profiles upon rechromatography (not shown). In contrast, alditol **3b** was quite weakly retarded (D 3.2), revealing only a single peak (Fig. 1I). The linear pentasaccharide, βGal →4βGlcNAc→3βGal→4βGlcNAc→6Gal (**4a**) (Fig. 1D; D1 14, D2 22), and its corresponding alditol (Fig. 1J; D 4.3) behaved very much like saccharides **2a** and **3a** and their alditols **2b** and **3b**, respectively. The more retarded peak fraction of Fig. 1D gave again the two-peak profile in a rechromatography experiment (not shown). The branched trisaccharide βGlcNAc→3(βGlcNAc→6)Gal (**5a**) had a stong affinity for WGA–agarose[4] (Fig. 1E), whereas alditol **5b** was very weakly bound (Fig. 1K). Unreduced **5a** gave an elution profile of two interconvertible[4] peaks (D1 16, D2 40), whereas **5b** gave only one peak (D 4.8). Overall, the branched trisaccharide **5a** and its alditol **5b** resembled very much disaccharide **1a** and its alditol **1b**, respectively. Even the branched pentasaccharide, βGal→4βGlcNAc→3(βGal→4βGlcNAc→6)Gal (**6a**), was very strongly bound to the lectin column, giving two peaks (Fig. 1F; D1 16, D2 41). The corresponding alditol **6b** was more weakly retained, giving only one single peak (Fig. 1L; D 10). Alditol **6b** was, however, retarded more than alditol **5b**, showing that the two non-

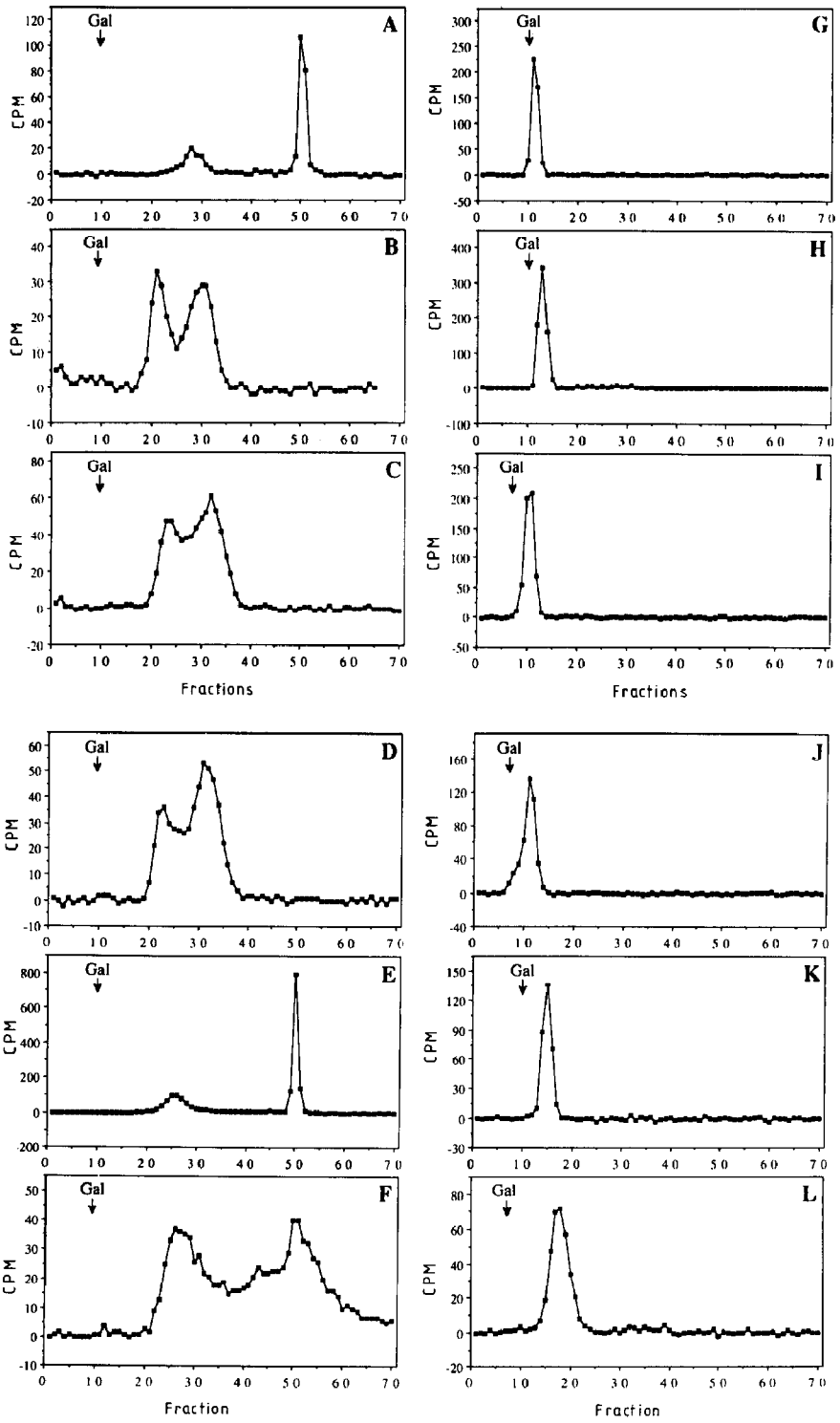

Fig. 1. WGA–agarose chromatography of radiolabeled samples of reducing oligosaccharides **1a–6a** and alditols **1b–6b**: (A) **1a**, (B) **2a**, (C) **3a**, D (**4a**), E (**5a**), (F) **6a**, (G) **1b**, (H) **2b**, (I) **3b**, (J) **4b**, (K) **5b**, and (L) **6b**. The arrow marked "Gal" shows the void of the column.

reducing D-galactosyl end-groups of **6b** enhanced the binding affinity significantly. The double-peak profiles of panels of Figs. 1 (A–F) have one thing in common; the slow peak was larger (56–70% of the total radioactivity) than the fast peak (30–44% of the total) for all six oligosaccharides.

WGA–agarose chromatography of N-*acetyllactosamine-type oligosaccharides bearing a 2-acetamido-2-deoxyglucose residue at the reducing end* (**7a–10a**); *comparison with the corresponding alditols* (**7b–10b**). — Another series of chromatograms run on the small column of WGA–agarose is collected in Fig. 2. The left-side panels (A–D) show the elution profiles obtained with unreduced oligosaccharides **7a–10a**, and the corresponding alditols **7b–10b** are shown in the right-side panels (E–H). Trisaccharide βGlcNAc→6βGal→4GlcNAc (**7a**), a cleavage product[4] from tetrasaccharide **9a** synthesized *in vitro*, was moderately retained (D 10) on WGA–agarose (Fig. 2A). It gave an elution profile of a single peak, behaving like the trisaccharide previously isolated from teratocarcinoma cells[1]. Alditol **7b** was bound more tightly (D 21) than unreduced **7a** (Fig. 2E). Linear tetrasaccharide, βGal→4βGlcNAc→6βGal→4GlcNAc (**8a**), was strongly bound and eluted as a single peak (Fig. 2B; D 18). The corresponding alditol **8b** was bound more tightly than unreduced **8a** (Fig. 2F; D 40). Tetrasaccharide **8a** and its alditol **8b** were more tightly bound to WGA than trisaccharide **7a** and its alditol **7b**, respectively (*cf.* panel B with A, and panel F with E in Fig. 2). In this case the nonreducing D-galactosyl end-group enhanced the binding affinity, in contrast to compound **2a**. Branched tetrasaccharide, βGlcNAc→3(βGlcNAc→6)βGal→4GlcNAc (**9a**), was rather weakly bound (D 3.6) to the lectin column[4] (Fig. 2C), showing a much smaller WGA affinity than branched trisaccharide **5a**. In turn, alditol **9b** was retained relatively strongly (Fig. 2G; D 14). Branched hexasaccharide, βGal→4βGlcNAc→3(βGal→4βGlcNAc→6)βGal→4GlcNAc (**10a**), was distinctly retarded by the WGA column (Fig. 2D; D 6.2). The corresponding alditol, **10b** was retained slightly more (Fig. 2H; D 7.9). It is remarkable that the nonreducing D-galactosyl end-groups enhanced the binding affinity of **10a** as compared to **9a**, but diminished that of the alditol **10b** as compared to that of the alditol **9b**.

Panels A–D show that the four saccharides bearing a 2-acetamido-2-deoxyglucose residue at the reducing end have one thing in common, *i.e.*, all gave elution profiles that contained only a single peak.

WGA–agarose chromatography of tetrasaccharide βGal→4βGlcNAc→6βGal→4Glc (**11a**); *comparison with the corresponding alditol* **11b**. — Tetrasaccharide **11a** was relatively strongly bound (Fig. 3A; D 25) like its 2-acetamido-2-deoxyglucose analog **8a** (*cf.* Fig. 2B). Alditol **11b** was even more strongly bound than the reducing tetrasaccharide **11a** itself. Accordingly, even alditol **11b** resembled its 2-acetamido-2-deoxyglucitol analog **8b**.

WGA–agarose chromatography of oligosaccharides bearing a 2-acetamido-2-deoxygalactose residue at the reducing end (**12a–15a**); *comparison with the corresponding alditols* **12b–15b**. — Radiolabeled disaccharide **12a**, βGlcNAc→6GalNAc, gave two peaks in WGA–agarose chromatography (Fig. 4A). The less retarded one (D1 16) was very broad, as if it consisted of several components having different binding

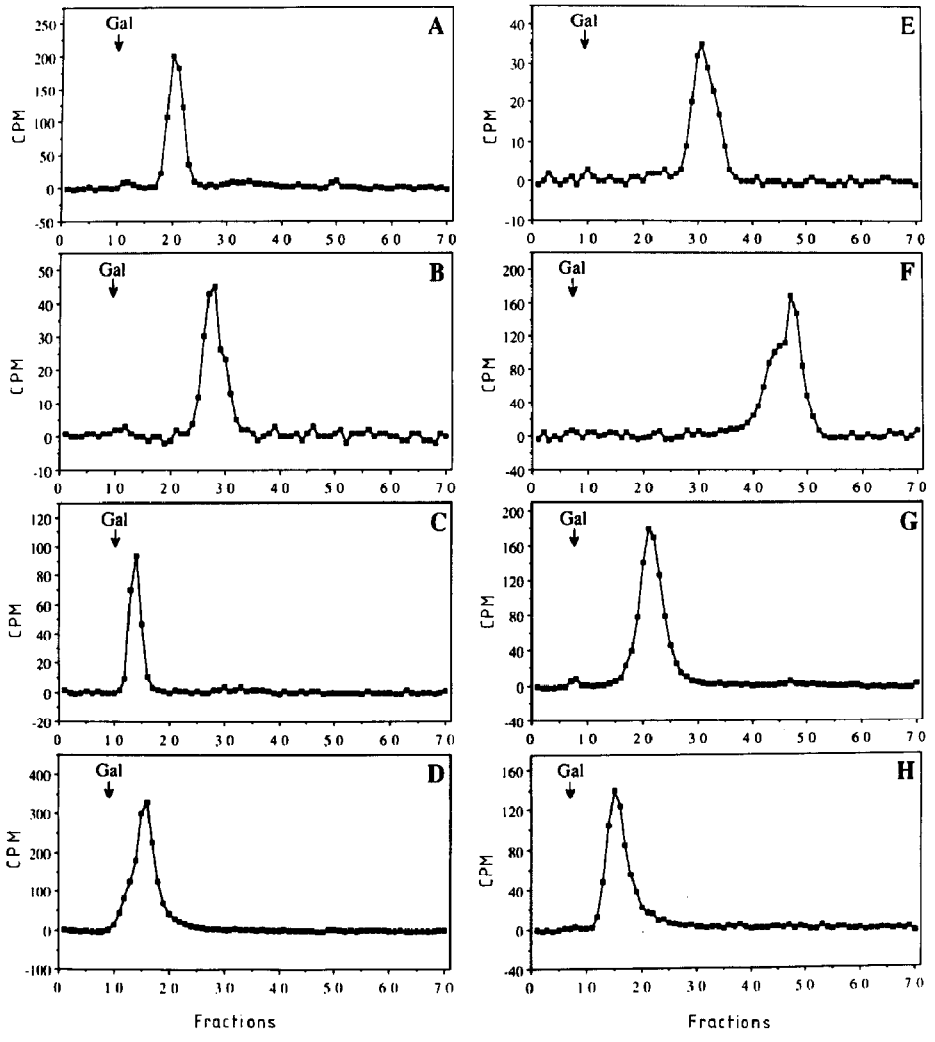

Fig. 2. WGA–agarose chromatography of radiolabeled samples of reducing oligosaccharides **7a**–**10a** and alditols **7b**–**10b**: (A) **7a**, (B) **8a**, (C) **9a**, (D) **10a**, (E) **7b**, (F) **8b**, (G) **9b**, and (H) **10b**.

affinities; the other fraction was quite strongly bound, being eluted as a sharp peak with 0.2M 2-acetamido-2-deoxy-D-glucose (D2 40). Overall, the elution pattern resembled rather closely that of βGlcNAc→6Gal (**1a**) (*cf.* Fig. 1A), suggesting that a 6-substituted 2-acetamido-2-deoxy-D-galactose and 6-substituted D-galactose residues have similar WGA-binding properties. This similarity between **12a** and **1a** was evident also in the chromatogram of the disaccharide alditol **12b** (Fig. 4C), which revealed a very small binding affinity to WGA (D 0.8), resembling closely that of the disaccharide alditol **1b** (*cf.* Fig. 1G). Radiolabeled trisaccharide, βGal→3(βGlcNAc→6)GalNAc (**13a**), was strongly bound to WGA–agarose, giving two peaks (Fig. 4B; D1 20, D2 39). The corresponding alditol **13b** was retarded only very slightly (Fig. 4D; D 0.65). The

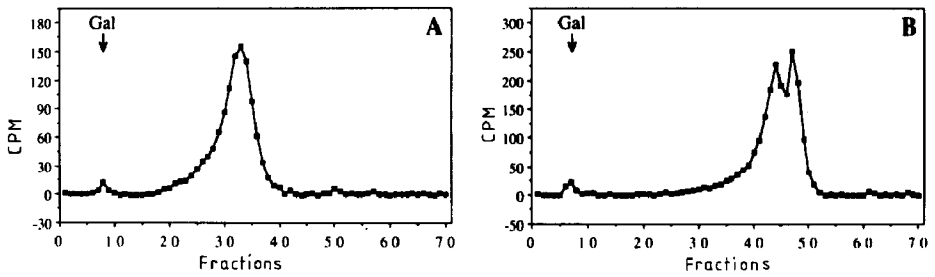

Fig. 3. WGA–agarose chromatography of radiolabeled samples of the reducing oligosaccharide **11a** (A) and of the alditol **11b** (B). The double peak around Fraction 45 (B) is an artifact caused by the addition of 0.2M 2-acetamido-2-deoxy-D-glucose to the eluent after 40 fractions had been collected with the sugar-free buffer.

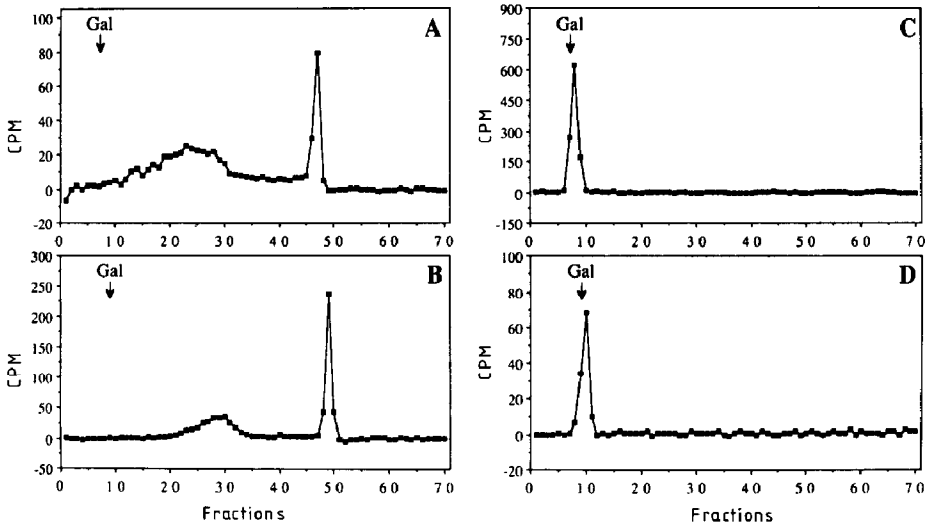

Fig. 4. WGA–agarose chromatography of radiolabeled samples of reducing oligosaccharides **12a** (A) and **13a** (B), and alditols **12b** (C) and **13b** (D).

behavior of this pair of saccharides resembled rather closely that of the branched trisaccharides **5a** and **5b** (see above). Upon rechromatography on WGA–agarose, both peak fractions of reducing **13a** gave again the original two-peak profile (not shown). In clear contrast to **12a** and **13a**, radiolabeled disaccharide **14a**, βGal→3GalNAc, and the corresponding alditol **14b** were not retarded in the WGA–agarose column. The different affinities of **12a** and **14a** and their corresponding alditols **12b** and **14b** suggest that WGA–agarose chromatography may be used in differentiating the core glycans of the mucin-type oligosaccharides of glycoproteins.

WGA–agarose chromatography of reduced chitooligosaccharides **15b** *and* **16b**. — βGlcNAc→4[1-^{3}H]GlcNAcol (^{3}H-**15b**) was retarded in the WGA–agarose column only slightly (D 3.3). The trisaccharide alditol, βGlcNAc→4βGlcNAc→4[1-^{3}H]GlcNAcol (^{3}H-**16b**), was strongly retarded (D 42–43). These data make it possible to compare, roughly, the relative binding affinities of the oligosaccharides of the present investigation with several compounds studied by Goldstein *et al.*[15] using precipitation-inhibition experiments.

DISCUSSION

The present WGA–agarose affinity data were obtained with saccharides synthesized enzymatically *in vitro*. They confirmed and extended the findings of our previous experiments[1-4] carried out with oligosaccharides obtained from naturally occurring materials.

Reducing oligosaccharides **1a–6a**, **12a**, and **13a** were strongly bound to WGA–agarose. All these compounds carried a D-galactose or 2-acetamido-2-deoxy-D-galactose residue at the reducing terminus. In marked contrast, alditols **1b–6b**, **12b**, and **13b**, derived from these oligosaccharides were retained very little by the lectin column (see Figs. 1 and 4). This finding suggests that the pyranose forms of the reducing D-galactose and 2-acetamido-2-deoxy-D-galactose residues are important for strong WGA binding. Previously, WGA had been regarded as rather specific for chitin oligosaccharides and their derivatives[15], although *N*-acetylneuraminic acid-containing saccharides[16,17], hybrid-type glycopeptides containing the βMan→4βGlcNAc→4βGlcNAc→NAsn structure[18], and asialoovine submaxillary mucin terminated by clustered arrays of αGalNAc→3Ser units[16,17] also have been known to possess distinct affinities for WGA.

The alditols **7b–11b** revealed consistently stronger WGA binding than the corresponding reducing oligosaccharides. Moreover, these alditols generally were more retarded than the alditols **1b–6b**, **12b**, and **13b**. Among the fifteen alditols (Table I), the total range of the D-values varied from 0.65 to 40. This wide range of WGA-affinities is remarkable, considering that the alditols were derived from a subgroup of structurally related oligosaccharides, all having high WGA-affinities. This suggests that the fractionation of *N*-acetyllactosaminooligosaccharides can be advantageously carried out by separating their alditols on small WGA–agarose columns.

A comparison of the linear tetrasaccharide **8a** and its glucose analog **11a**, and their alditols **8b** and **11b**, is interesting because of the great similarities in the WGA affinities. The strong WGA-binding of the glucose-containing **11a** suggests that some glycolipids may be bound to the lectin through this "epitope". The presence of consistent structure–affinity relationships, like in the few examples cited above, adds to the value of WGA–agarose chromatography of *N*-acetyllactosaminooligosaccharides and their alditols.

Unlike the abovementioned examples, some structural changes did not cause consistent affinity changes. For example, oligosaccharides **7a**, **9a**, and **10a**, which carry a 2-acetamido-2-deoxy-D-glucose residue at the reducing end, had notably smaller affin-

ities than the related oligosaccharides **1a**, **5a**, and **6a**, which carry a D-galactose residue as a reducing end. However, the affinity of **8a** was, unexpectedly, larger than that of the faster peak of **2a**. Another example of variable affinity changes was provided by the effect of introducing a nonreducing D-galactosyl-end group. For instance in alditol **6b**, the nonreducing D-galactosyl groups enhanced the affinity as compared to that of alditol **5b**, but in alditol **10b** they lessened the affinity as compared to that of alditol **9b**. These few examples of inconsistent structure-affinity relationship suggest that much remains to be learned about the WGA-binding of *N*-acetyllactosaminooligosaccharides.

The strongly-bound, reducing oligosaccharides **1a–6a**, **12a**, and **13a** gave elution profiles of double peaks. In several experiments, described here and elsewhere[2,4], where the two fractions were separately recovered, desalted by ion exchange, and rechromatographed on WGA–agarose, they consistently gave again the original two-peak profiles. This suggests that the two peaks represent interconvertible oligosaccharides; possibly mutarotational isomers. The present data showing that the corresponding alditols **1b–6b**, **12b**, and **13b** gave only single peaks suggested that the intact reducing-end residue is important for peak splitting. The alditol chromatograms also evidenced the homogeneity of the original oligosaccharides despite the two peaks. Thus, they lend further support that a slow mutarotation may indeed have caused the peak splitting in the original chromatography of **1a–6a**, **12a**, and **13a**. All these oligosaccharides carried a D-galactose or 2-acetamido-2-deoxy-D-galactose residue at the reducing end.

We do not know of any reported observations, analogous to ours, which would suggest that slow mutarotation can cause partial separation of isomeric forms of a given saccharide on lectin-affinity columns. However, we have made an analogous finding in the chromatography of radiolabeled oligosaccharides in a column of immobilized *Griffonia simplicifolia* I isolectins. Radioactive D-galactose appeared to be separated into two peaks in this system[19] that is capable of separating α- and β-glycosides of D-galactose[20]. This observation lends further support to our working hypothesis, which seeks to explain the peak splitting of oligosaccharides **1a–6a**, **12a**, and **13a** by postulating different WGA-affinities of mutarotational isomers and slow kinetics of mutarotation under the conditions of WGA-chromatography. Obviously, this hypothesis is largely based on indirect evidence and it must be tested by more direct experimenting in the future. Assuming that the mutarotation equilibria of the strongly bound oligosaccharides **1a–6a** resemble that of free D-galactose, we may make a guess about the nature of the two peaks in Figs. 1A–F as follows. Aqueous equilibrium solutions of D-galactose were shown to consist of β-pyranose (63.9%), α-pyranose (32.0%), β-furanose (3.1%), and α-furanose (1.0%) forms[21]; the open-chain aldehyde may amount to only 0.097%[22]. Accordingly, it appears possible that the slow components (which are preponderant) in the elution profiles of **1a–6a** represent the β-pyranose forms of the oligosaccharides, and the fast components may represent the α-pyranose forms.

Among the saccharides of high WGA affinity, the disaccharide, βGlcNAc→6GalNAc (**12a**), and the branched trisaccharide, βGal→3(βGlcNAc→6)GalNAc (**13a**), are interesting because they are common components in the glycoprotein glycan chains that

are *O*-linked to serine or threonine residues of glycoproteins. The present data show that the strongly-bound forms of these reducing oligosaccharides have affinities approaching that of reduced *N,N',N''*-triacetylchitotriose (**16b**). It is remarkable that no previous reports have appeared describing the WGA-affinity of glycopeptides that carry these glycan chains; this may imply that the strongly bound "mutarotational form" of 2-acetamido-2-deoxy-D-galactose is not present in natural glycoproteins.

If an extra 2-acetamido-2-deoxy-D-glucose or D-glucose residue is "added" to the reducing end of oligosaccharides **1a**, **2a**, or **5a**, **6a**, the resulting WGA-chromatograms revealed only one single peak; no peak splitting occurred (see Figs. 2 and 3). This suggests that the mutarotational forms of 2-acetamido-2-deoxy-D-glucose, and those of D-glucose, play a role different from that of D-galactose in WGA-binding.

The relatively small retardation observed in WGA chromatography of radiolabeled βGlcNAc→4GlcNAcol (**15b**) and the strong binding observed for βGlcNAc→4 βGlcNAc→4GlcNAcol (**16b**) can be compared with that of the fourteen oligosaccharides of oligo-*N*-acetyllactosamine type and their alditols shown in Table 1. This comparison shows that most of the oligosaccharides and alditols studied in the present experiments bind to WGA more strongly than **15b** but more weakly than **16b**. The affinity of the reduced chitobiose **15b**, in turn, has been compared to several other oligosaccharides in the WGA-binding studies of Goldstein *et al.*[15]

The individual isolectin forms of WGA[23] may interact differently with the oligosaccharides, thus causing some variation in the binding properties of different WGA preparations. Having reported observations[24] concerning the high affinity of βGal→4βGlcNAc→6Gal (**2a**) for WGA, Osawa *et al.*[25] also have studied more recently WGA-chromatography of reduced *N*-acetyllactosaminooligosaccharides with success.

REFERENCES

1 O. Renkonen, P. Mäkinen, K. Hård, J. Helin, and L. Penttilä, *Biochem. Cell Biol.*, 66 (1988) 449–453.
2 O. Renkonen, *Abstr. Int. Carbohydr. Symp., XIVth*, (1988) c-32.
3 O. Renkonen, L. Penttilä, A. Makkonen, R. Niemelä, A. Leppänen, J. Helin, and A. Vainio, *Glycoconjugate J.*, 6 (1989) 129–140.
4 A. Seppo, L. Penttilä, A. Makkonen, A. Leppänen, R. Niemelä, J. Jäntti, J. Helin, and O. Renkonen, *Biochem. Cell Biol.*, 68 (1990) 44–53.
5 K. Brew, T. C. Vanaman, and R. L. Hill, *Proc. Nat. Acad. Sci. U.S.A.*, 59 (1968) 491–497.
6 C. Hughes and R. W. Jeanloz, *Biochemistry*, 3 (1964) 1535–1543.
7 J. C. Paulson, J.-P. Prieels, L. R. Glasgow, and R. L. Hill, *J. Biol. Chem.*, 253 (1978) 5617–5624.
8 O. Renkonen, J. Helin, A. Vainio, R. Niemelä, L. Penttilä, and P. Hilden, *Biochem. Cell Biol.*, 68 (1990) 1032–1036.
9 L. Hough, *Methods Carbohydr. Chem.*, 5 (1965) 370–377.
10 I. Brockhausen, D. Williams, K. L. Matta, J. Orr, and H. Schachter, *Can. J. Biochem. Cell Biol.*, 61 (1983) 1322–1333.
11 M.-L. Rasilo and O. Renkonen, *Hoppe Seyler's Z. Physiol. Chem.*, 363 (1982) 89–93.
12 R. G. Spiro, *Methods Enzymol.*, 28B, (1972) 3–43.
13 A. Leppänen, A. Korvuo, K. Puro, and O. Renkonen, *Carbohydr. Res.*, 153 (1986) 87–95.
14 H. Yamaguchi, S. Inamura, and K. Makino, *J. Biochem. (Tokyo)*, 79 (1976) 299–303.
15 I. J. Goldstein, S. Hammarström, and G. Sundblad, *Biochim. Biophys. Acta*, 405 (1975) 63–67.
16 B. P. Peters, S. Ebisu, I.J. Goldstein, and M. Flashner, *Biochemistry*, 18 (1979) 5505–5511.
17 V. P. Bhavanandan and A. W. Katlic, *J. Biol. Chem.*, 254 (1979) 4000–4008.
18 K. Yamamoto, T. Tsuji, I. Matsumoto, and T. Osawa, *Biochemistry*, 20 (1981) 5894–5899.

19 O. Renkonen, unpublished observations.
20 D. A. Blake and I. J. Goldstein, *Anal. Biochem.*, 102 (1980) 103–109.
21 T. E. Acree, R. S. Shallenberger, C. Y. Lee, and J. W. Einset, *Carbohydr. Res.*, 10 (1969) 355–360.
22 P. O. I. Virtanen, E. Oikarinen, and H. Nevala, *Abstr. Int. Carbohydr. Symp., XIVth*, (1988) A-110.
23 R. H. Rice and M. E. Etzler, *Biochemistry*, 14 (1975) 4093–4099.
24 T. Osawa, H. Kawashima, Y. Yamashita, K. Yamamoto, and Y. Imai, *Abstr. Int. Symp. Glycoconjugates, Xth*, (1989) 58.
25 H. Kawashima, S. Sueyoshi, H. Li, K. Yamamoto, and T. Osawa, *Glycoconjugate J.*, 7 (1990) 323–334.