The structural determinants required for interaction of oligosaccharides with *Ricinus communis* agglutinin I (RCA) and *Ricinus communis* agglutinin II (RCAII) have been studied by lectin affinity high-performance liquid chromatography (HPLC). Homogeneous oligosaccharides of known structure, purified following release from Asn with N-glycanase and reduction with NaB\(\text{H}_4\) or NaBH\(_4\), were tested for their ability to interact with columns of silica-bound RCA and RCAII. The characteristic elution position obtained for each oligosaccharide was reproducible and correlated with specific structural features. RCAI binds oligosaccharides bearing terminal \(\beta 1,4\) -linked Gal but not those containing terminal \(\beta 1,4\) -linked GalNAc. In contrast, RCAII binds structures bearing \(\beta 1,4\)-linked Gal or \(\beta 1,4\)-linked GalNAc. Both lectins display a greater affinity for structures with terminal \(\beta 1,4\)-rather than \(\beta 1,3\)-linked Gal, although RCAII interacts more strongly with RCA, with oligosaccharides containing terminal \(\beta 1,3\)-linked Gal. Whereas terminal \(\alpha 2,6\)-linked sialic acid partially inhibits oligosaccharide-RCAI interaction, terminal \(\alpha 2,3\)-linked sialic acid abolishes interaction with the lectin. In contrast, \(\alpha 2,3\)- and \(\alpha 2,6\)-linked sialic acid equally inhibit but do not abolish oligosaccharide interaction with RCAII. RCAI and RCAII discriminate between \(N\)-acetyllactosamine-type branches arising from different core Man residues of dibranched complex-type oligosaccharides; RCAI has a preference for the branch attached to the \(\alpha 1,3\)-linked core Man and RCAII has a preference for the branch attached to the \(\alpha 1,6\)-linked core Man. RCAI but not RCAII interacts with certain di- and tribranched oligosaccharides devoid of either Gal or GalNAc but bearing terminal GlcNAc, indicating an important role for GlcNAc in RCAII interaction. These findings suggest that \(N\)-acetyllactosamine is the primary feature required for oligosaccharide recognition by both RCAI and RCAII but that lectin interaction is strongly modulated by other structural features. Thus, the oligosaccharide specificities of RCAI and RCAII are distinct, depending on many different structural features including terminal sugar moieties, peripheral branching pattern, and sugar linkages.

The common castor bean, *Ricinus communis*, contains two lectins, *Ricinus communis* agglutinin I (RCAI) and *Ricinus communis* agglutinin II (RCAII). RCAI (RCA-120) is a tetrameric hemagglutinin with a molecular weight of 120,000, consisting of two \(\alpha (M_r = 29,500)\) and two \(\beta (M_r = 37,000)\) subunits, RCAII (RCA-60) is a highly toxic, dimeric protein with a molecular weight of 60,000, consisting of a single \(\alpha (M_r = 29,500)\) and a single \(\beta (M_r = 34,000)\) subunit. For both lectins, only the \(\beta\) subunit displays carbohydrate binding activity. Thus RCAI, which is essentially monovalent with only one \(\beta\) subunit, is not able to agglutinate cells, whereas RCAII, which is divalent with two \(\beta\) subunits, is a true agglutinin. The constituent polypeptide subunits of RCAI and RCAII have closely related antigenic features and amino acid sequences (for review see Refs. 1–4).

In an earlier study, we systematically examined the affinities of RCAI and RCAII for glycopeptides bearing either Asn-linked or O-glycosidically linked oligosaccharides (3). RCAI and RCAII were found to each display a high degree of specificity for oligosaccharide structural features. Subsequent studies from a number of laboratories using various techniques have further established the specificities of these two lectins (2, 4–6). We have recently developed a technique of lectin affinity high-performance liquid chromatography (HPLC) for analyzing and separating oligosaccharides and have established the utility of such a method for defining lectin specificity (7). Utilizing homogeneous N-glycanase-released, reduced oligosaccharides of known structure, we have examined the oligosaccharide specificities of RCAI and RCAII by lectin affinity HPLC. These studies have provided significant new insights about the specificity of RCAI and RCAII. The elution position of individual oligosaccharides during RCAI and RCAII affinity HPLC is reproducible and highly characteristic of specific structural features. Therefore, lectin affinity HPLC using RCAI and RCAII is a useful adjunct for the fractionation and characterization of oligosaccharide structures.

**MATERIALS AND METHODS**

**Oligosaccharide Standards**—The oligosaccharides used in this study were released from protein by digestion with N-glycanase, reduced with either NaB\(\text{H}_4\) or NaBH\(_4\), and characterized as described in the accompanying paper (8). Their structures are shown in Table I. Glycoproteins were obtained from the sources given in the accompanying manuscript (8), except for bovine Factor X, which was a gift from Dr. Charles Esmon (Oklahoma Medical Research Foundation). Sources of the oligosaccharide standards were as follows: Di-1 from human transferrin (9), Di-2 and Di-3 from human chorionic gonadotropin (10–12), Di-4 and Di-7 from bovine gammaglobulin.

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1 The abbreviations used are: RCAI, *Ricinus communis* agglutinin I; RCAII, *Ricinus communis* agglutinin II; L-PHA, leukoagglutinating phytohemagglutinin; E-PHA, erythroagglutinating phytohemagglutinin; HPLC, high-performance liquid chromatography; Sia, sialic acid; SO4, sulfate; PBS, phosphate-buffered saline.
Factor was removed from oligosaccharides by digestion in 0.2 BW5147 cells (8, 19), Tetra-1 from human orosomucoid (20), and with exoglycosidases or glycosyltransferases, as indicated.

Cacodylate (pH 6.0) with either affinity purified Clostridium HM-1 from IgM myeloma protein (21, 22). Additional oligosaccharide affinity HPLC have recently been described.

were released from oligosaccharides by digestion with Diplococcal lectin affinity HPLC columns were run in PBS/Na3 (6.7 mM KH2PO4, 0.15 M NaCl, 0.02% NaN3, pH 7.4) at 0.5 ml/min. Oligosaccharides bound by RCAI were eluted with 0.5 ml (1 min) of 200 mM lactose in PBS/Na3, while those bound by RCAII were eluted with 10.0 ml (20 min) of 100 mM GalNAc and 1.0 N acetic acid in PBS/Na3.

RESULTS

Purified N-glycanase-released, reduced oligosaccharides with the structures shown in Table I and products derived from these oligosaccharides were examined by lectin affinity HPLC on silica-bound RCAI and RCAII. Results of such analyses are shown in Figs. 1-8, along with schematically illustrated structures of the oligosaccharides examined. Although reproducible elution times characteristic of specific oligosaccharide structures were obtained with both RCAI- and RCAII-silica columns, we have presented the data in terms of elution within discrete regions (I-V for RCAI and I-IV for RCAII). In each case, region I corresponds to the column void, i.e. complete lack of interaction with the immobilized lectin. Polysaccharides which are not recognized by either RCAI or RCAII are structurally unrelated to Asn-linked oligosaccharides but of similar size, for example, (GlcNAcβ1,4)5 elute in region 1. Oligosaccharides which interact with RCAI- or RCAII-silica are retarded to varying extents by the lectins and in some cases require highly stringent conditions for elution.

Analysis of Oligosaccharides by RCAI Affinity HPLC—Oligosaccharides elute within five major regions (I-V) during RCAI affinity HPLC. As noted above, region I corresponds to the column void, while regions II-IV reflect a progressive increase in interaction with RCAI that results in retardation but not binding of oligosaccharides. Structures bound by RCAI-silica remain tightly associated with the lectin without evidence of elution for >60 min of chromatography in PBS/Na3. Bound oligosaccharides were eluted at 45 min (region V) with 200 mM lactose. Neither high concentrations of other sugars, including Gal, GlcNAc, and GalNAc, nor acidic pH (1.0 N acetic acid) were effective at eluting bound oligosaccharides.

Dibranched complex-type oligosaccharides bearing terminal α2,3- versus α2,6-linked sialic acid differ in their interaction with RCAII. Oligosaccharides bearing 2 α2,6-linked sialic acid residues (Di-1) elute in region II (Fig. 1A), whereas those bearing 2 α2,3-linked sialic acid moieties (Di-2) elute retarded in region I (Fig. 1B). A minor subpopulation of Di-1 oligosaccharides consists of dibranched structures containing 1 residue each of α2,3- and α2,6-linked sialic acid, as assessed by differential digestion with Newcastle disease virus versus C. perfringens neuraminidase (8). The latter structures elute as a small peak between regions I and II (Fig. 1A).

Removal of sialic acid from dibranched complex-type oligosaccharides, which exposes the underlying β1,4-linked Gal residues, results in binding to RCAI. Thus, asialo Di-1 (data not shown) and asialo Di-2 (Fig. 1C) bind to the lectin and...
### RCA\(_i\) and RCA\(_{ii}\) Affinity HPLC

**TABLE I**

**Structures of oligosaccharides analyzed by lectin affinity chromatography**

| Oligosaccharide Structure | Designation |
|---------------------------|-------------|
| Siaα2,6Galβ1,4GlcNAcβ1,2Manβ1 | D1-1 |
| Siaα2,6Galβ1,4GlcNAcβ1,2Manβ1 | D1-2 |
| Siaα2,6Galβ1,4GlcNAcβ1,2Manβ1 | D1-3 |
| Siaα2,6Galβ1,4GlcNAcβ1,2Manβ1 | D1-4 |
| Siaα2,6Galβ1,4GlcNAcβ1,2Manβ1 | D1-5 |
| SO₄GalNAcβ1,4GlcNAcβ1,2Manβ1 | D1-6 |
| Siaα2,6Galβ1,4GlcNAcβ1,2Manβ1 | D1-7 |
| Siaα2,6Galβ1,4GlcNAcβ1,2Manβ1 | D1-8 |
| Siaα2,6Galβ1,4GlcNAcβ1,2Manβ1 | D1-9 |

| Oligosaccharide Structure | Designation |
|---------------------------|-------------|
| Siaα2,6Galβ1,4GlcNAcβ1,2Manβ1 | D1-1 |
| Siaα2,6Galβ1,4GlcNAcβ1,2Manβ1 | D1-2 |
| Siaα2,6Galβ1,4GlcNAcβ1,2Manβ1 | D1-3 |
| Siaα2,6Galβ1,4GlcNAcβ1,2Manβ1 | D1-4 |
| Siaα2,6Galβ1,4GlcNAcβ1,2Manβ1 | D1-5 |
| SO₄GalNAcβ1,4GlcNAcβ1,2Manβ1 | D1-6 |
| Siaα2,6Galβ1,4GlcNAcβ1,2Manβ1 | D1-7 |
| Siaα2,6Galβ1,4GlcNAcβ1,2Manβ1 | D1-8 |
| Siaα2,6Galβ1,4GlcNAcβ1,2Manβ1 | D1-9 |
require 200 mM lactose for elution in region V. Subsequent removal of Gal completely abolishes interaction of dibranched oligosaccharides with RCAI, resulting in elution of the agalacto structures in region I (Fig. 1D). Thus, the presence of terminal β1,4-linked Gal promotes binding of oligosaccharides to RCAI. The presence of α2,6-linked sialic acid markedly reduces oligosaccharide-RCAI interaction, resulting in retardation but not binding of dibranched oligosaccharides to the lectin, whereas the presence of α2,3-linked sialic acid or absence of β1,4-linked Gal abolishes interaction of dibranched structures with RCAI. The core structure, consisting of ManαGlcNAcβ2, also does not interact with RCAI. Other oligosaccharides devoid of Gal and structurally distinct from complex-type oligosaccharides, for example high mannose-type structures (HM-1), also do not interact with RCAI (Fig. 1H).

Di-1 and Di-2 oligosaccharides differ both in the linkage of their sialic acid residues (α2,3 versus α2,6) and in the presence of Fuc on a large fraction of structures constituting Di-2 but not Di-1. The presence of Fuc attached to the reduced core GlcNAc does not appear to affect interaction of Di-2 with RCAI, since roughly half of the Di-2 oligosaccharides contain Fuc, yet analysis of Di-2 or structures derived from Di-2 by RCAI affinity HPLC consistently yields a single uniform peak. 2) desialylated forms of Di-1 and Di-2 coelute during RCAI affinity HPLC, yet only asialo Di-2 contains Fuc, and 3) removal of Fuc by α-fucosidase does not alter the interaction of Di-2 or structures derived from Di-2 with RCAI.

Dibranched complex-type oligosaccharides bearing a single terminal β1,4-linked Gal differ in their interaction with RCAI, depending upon the location of the branch bearing the peripheral Gal. Oligosaccharides with a single terminal β1,4-linked Gal on the branch arising from the α1,3-linked core Man (asialo Di-3) elute in region IV (Fig. 1E). In contrast, structures with a single terminal β1,4-linked Gal on the branch arising from the α1,6-linked core Man (Di-4) elute in region III (Fig. 1F). Removal of Gal from either asialo Di-3 or Di-4 abolishes interaction with RCAI, resulting in elution of the agalacto structures in region I. Thus, the affinity of RCAI for oligosaccharides bearing a single terminal β1,4-linked Gal is greater when this moiety is present on the branch attached to the α1,3- as compared to the α1,6-linked core Man. Of note, dibranched oligosaccharides containing a single terminal β1,4-linked Gal (asialo Di-3 and Di-4) are retarded but not bound by RCAI-silica, whereas dibranched structures bearing 2 terminal β1,4-linked Gal residues (asialo Di-1 and asialo Di-2) are tightly bound by the lectin.

Dibranched complex-type oligosaccharides containing β1,4-linked GalNAc in place of β1,4-linked Gal do not significantly interact with RCAI. Di-6, which bears two branches terminating in β1,4-linked SO4-GalNAc, elutes in region I during RCAI affinity HPLC (data not shown). Desulfated Di-6, which is identical to asialo Di-2 aside from the presence of terminal β1,4-linked GalNAc in place of β1,4-linked Gal, is slightly retarded by RCAI (Fig. 1G). The latter result indicates that the presence of an N-acetyl- rather than an HO moiety at the C-2 position of terminal β1,4-linked Gal results in a profound decrease in interaction with RCAI.

Dibranched oligosaccharides such as Di-5 contain one branch with the sequence SO4-GalNAcβ1,4GlcNAcβ1,2-Manα1,3 and one branch with the sequence SiaαGal-β1,4GlcNAcβ1,2Manα1,6. The interaction of Di-5 structures with RCAI depends upon the linkage of the sialic acid residue. Di-5 oligosaccharides containing an α2,6-linked sialic acid elute between regions I and II, while those bearing an α2,3-linked sialic acid elute in region I, as assessed by differential digestion with Newcastle disease virus versus C. perfringens neuraminidase (9). Asialo Di-5 coelutes with Di-4 in region III, while agalacto Di-5 elutes in region I. The behavior of Di-5 and structures derived from Di-5 demonstrates that the branch terminating in SO4-GalNAc does not affect the interaction of RCAI with the branch bearing the β1,4-linked Gal.

The interaction of RCAI with dibranched complex-type oligosaccharides also depends upon the linkage of terminal Gal moieties. Di-8 is identical to asialo Di-2 except for the presence of a terminal β1,3- rather than a β1,4-linked Gal on the branch arising from the α1,3-linked core Man. Like asialo Di-2, Di-8 binds to RCAI and is eluted in region V with 200 mM lactose (Fig. 2A). Removal of terminal β1,4-linked Gal (on the branch arising from the α1,6-linked core Man) completely abolishes interaction of Di-8 with RCAI (Fig. 2B). Thus, in contrast to the presence of a single terminal β1,4-linked Gal residue (Fig. 1E), the presence of a single terminal β1,3-linked Gal residue on the branch arising from the α1,3-linked core Man does not result in detectable interaction with RCAI. Di-9, which bears two terminal β1,3-linked Gal residues, is slightly retarded during RCAI affinity HPLC, eluting at the beginning of region II (Fig. 2C). Asialo Di-2, desulfated Di-6, and Di-9 share identical oligosaccharide structures except for the terminal moieties on the peripheral branches, bearing β1,4-linked Gal, β1,4-linked GalNAc, and β1,3-linked Gal, respectively. RCAI discriminates between these closely related oligosaccharides, binding the first structure and differentially retarding the second and third. The interaction with RCAI appears to be slightly greater for terminal β1,3-linked Gal than for terminal β1,4-linked GalNAc moieties.

The presence of a bisecting GlcNAc residue attached to the β-linked core Man does not appear to alter the behavior of...
dibranched complex-type oligosaccharides during RCA1 affinity HPLC. Di-7, which contains two α2,6-linked sialic acid residues, elutes in region II, whereas asialo Di-7 binds to RCA1 and is eluted in region V with 200 mM lactose (data not shown). Agalacto Di-7 elutes in region I. Thus, the analogous forms of Di-1 and Di-7 display the same interactions with RCA1, despite the presence of a bisecting GlcNAc on Di-7 but not Di-1.

Tetra-1 and Tri-1 each consist of a heterogeneous mixture of oligosaccharides which contain the same number of sialic acid residues (4 and 3, respectively) and share the identical underlying carbohydrate structure (Table I). Tetra-1 and Tri-1 oligosaccharides differ in the linkage (α2,3 versus α2,6) of sialic acid residues and in the distribution of α2,3- and α2,6-linked sialic acid moieties among the peripheral branches (8). Both Tetra-1 (Fig. 3A) and Tri-1 (Fig. 3B) separate into two peaks during RCA1 affinity HPLC. In each case, a major oligosaccharide fraction binds to the lectin and is eluted in region V, while the remainder elutes in region I or between regions I and II. These fractionations reflect the linkage and/or distribution of differently linked sialic acid moieties on Tetra-1 and Tri-1 oligosaccharides, since 1) analysis of each isolated Tetra-1 and Tri-1 fraction by differential digestion with Newcastle disease virus versus C. perfringens neuraminidase (8). Sialic acid residues were quantitatively released by Newcastle disease virus neuraminidase, whereas only 20% of the sialic acid residues on structures in the bound peak (region V) were released by this enzyme (8). Sialic acid residues were quantitatively released from oligosaccharides in both peaks by digestion with C. perfringens neuraminidase. Similar results were obtained by analysis of the earlier versus later eluting Tetra-1 oligosaccharides. Therefore, Tri-1 and Tetra-1 oligosaccharides bound by RCA1 contain relatively more α2,6-linked sialic acid (Newcastle disease virus neuraminidase resistant) than those structures not bound by RCA1; and 2) removal of sialic acid (Fig. 3C) or both sialic acid and Gal (Fig. 3D) from Tetra-1 and Tri-1 results in elution of the oligosaccharides as single, uniform peaks. Asialo Tetra-1 and asialo Tri-1 bind tightly to RCA1 (Fig. 3C), whereas agalacto Tetra-1 and agalacto Tri-1 elute unretarded in region I (Fig. 3D).

The sialylated forms of Tetra-1 and Tri-1 oligosaccharides not bound by RCA1 differ slightly in elution position (region I versus between regions I and II). This may reflect differences in linkage of sialic acid residues, distribution of distinctly linked sialic acid moieties among the peripheral branches, number of peripheral branches (four versus three), and/or the presence of a branch arising from the C-6 position of the α1,6-linked core Man on Tetra-1 but not Tri-1. Due to the limited knowledge of the detailed structures of these heterogeneously sialylated oligosaccharides, these possibilities cannot at present be discriminated. Finally, in contrast to sialylated dibranched structures, which are at best retarded but not bound by RCA1 (Fig. 1A), the presence of three or four sialylated branches is in some cases sufficient for binding of oligosaccharides to RCA1 (Fig. 3A and B).

Additional mono- and disaccharide derivatives were also examined by RCA1 affinity HPLC. [3H]Gal and [3H]UDP-Gal both elute in region I (2 min). In contrast, reduced [3H]lactose (Galβ1,4Glc) elutes between regions I and II (5 min), while reduced [3H]lactosamine (Galβ1,4GlcNAc) elutes at the beginning of region II (8 min). Notably, unreduced [3H]lactosamine elutes in the early portion of region IV (21 min). Thus, it appears that RCA1 discriminates among penultimate sugar moieties, with GlcNAc being preferred over reduced GlcNAc or reduced Gal. We would predict that lactose would interact with RCA1 to a greater degree than reduced lactose. Furthermore, the ability of lactose but not Gal to elute bound oligosaccharides from RCA1-silica can be readily understood.

Analysis of Oligosaccharides by RCA1 Affinity HPLC—Oligosaccharides elute within four major regions (I-IV) during RCA1 affinity HPLC. Region I again corresponds to the column void, while region II reflects oligosaccharides retarded but not bound by RCA1. Regions III and IV correspond to differing elution positions of bound oligosaccharides. Structures bound by RCA1 remain tightly associated with the lectin, with no evidence of elution for >60 min of chromatography in PBS/Na3. Bound oligosaccharides were eluted at 30–40 min with 100 mM GalNAc in 1.0 N acetic acid. These conditions were the only ones found to effectively and quantitatively elute RCA1-bound oligosaccharides. For example, high concentrations of GalNAc, Gal, GlcNAc, or lactose in the absence of acetic acid, acetic acid alone, or high concentrations of sugars other than GalNAc in acetic acid did not result in elution of bound oligosaccharides from the lectin.

The presence of α2,3- versus α2,6-linked sialic acid does not appear to differentially affect oligosaccharide interaction with RCA1. Di-1 and Di-2, which contain two terminal α2,6- and α2,3-linked sialic acid moieties, respectively, coelute in
region II during RCAII affinity HPLC (Fig. 4, A and B). The asialo forms of Di-1 and Di-2, each bearing two terminal β1,4-linked Gal residues, bind to the lectin and elute between regions III and IV following addition of 200 mM GalNAc in 1.0 N acetic acid (Fig. 4C). Removal of Gal from these di-branched structures reduces but does not abolish interaction with RCAII. Agalacto Di-1 and agalacto Di-2 elute in region II, slightly earlier than the sialylated forms of these oligosaccharides (Fig. 4D). Removal of terminal GlcNAc from the agalacto structures abolishes interaction with RCAII, resulting in elution of the ManβGlcNAc core in region I (Fig. 4E). The presence of Fuc attached to the reduced core GlcNAc does not appear to affect interaction of di-branched oligosaccharides with RCAII, for the same reasons cited above for RCAI.

The presence of a bisecting GlcNAc alters the interaction of di-branched complex-type oligosaccharides with RCAII. Whereas Di-7 coelutes with Di-1 and Di-2 in region II (data not shown), asialo Di-7 elutes in region III, earlier than asialo Di-1 and asialo Di-2 (Fig. 4F). Furthermore, agalacto Di-7 elutes in region I (Fig. 4G) in contrast to agalacto Di-1 and agalacto Di-2, which elute in region II (Fig. 4D). Thus, desialylated di-branched oligosaccharides bearing a bisecting GlcNAc display diminished interaction with RCAII.

Interaction of RCAII is stronger with oligosaccharides bearing a peripheral branch arising from the α1,6- rather than α1,3-linked core Man. Di-3 and α2,3-sialylated Di-4 (8) contain a single peripheral branch with the sequence Siaα2,3-Galβ1,4GlcNAcβ1,2 attached to α1,3- and α1,6-linked core Man residues, respectively. Di-3 elutes in region I (Fig. 5A), whereas α2,3-sialylated Di-4 coelutes with Di-1 and Di-2 in region II (Fig. 5B). Thus, structures bearing a single sialylated branch attached to the α1,6-linked core Man (α2,3-sialylated Di-4) display identical behavior during RCAII affinity HPLC as structures bearing two sialylated branches (Di-1 and Di-2). Asialo Di-3 is retarded but not bound by the lectin, eluting in region II (Fig. 5C), whereas Di-4 is bound by RCAII, eluting in region III (Fig. 5D). Thus, for both sialylated and desialylated di-branched oligosaccharides, RCAII displays a greater affinity for the peripheral branch attached to the α1,6-linked core Man. Furthermore, in contrast to the corresponding
sialylated structures, asialo Di-1 and asialo Di-2 do not coelute with Di-4, indicating that oligosaccharide interaction with RCAII is enhanced by the presence of a second terminal β1,4-linked Gal residue.

The behavior of dibranched complex-type oligosaccharides during RCAII affinity HPLC is also influenced by the linkage of Gal residues. Di-8, which contains one terminal β1,3- and one terminal β1,4-linked Gal, elutes in region IV (Fig. 6A), later than asialo Di-1 and asialo Di-2, both of which bear two terminal β1,4-linked Gal moieties. Removal of the β1,4-linked Gal residue from Di-8 slightly reduces interaction of the oligosaccharide with RCAII, resulting in coelution with asialo Di-1 and asialo Di-2 between regions III and IV (Fig. 6B). Complete removal of the Galβ1,4GlcNAcβ1,2 peripheral branch significantly reduces but does not eliminate the interaction of Di-8 with RCAII (Fig. 6C). Di-9, which bears two terminal β1,3-linked Gal residues, elutes late in region III (Fig. 6D), slightly earlier than dibranched structures bearing two terminal β1,4-linked Gal moieties (asialo Di-1 and asialo Di-2).

Dibranched oligosaccharides bearing the peripheral sequence SO4-GalNAcβ1,4GlcNAcβ1,2 display the same interaction with RCAII as dibranched structures bearing the peripheral sequence SiaαGalβ1,4GlcNAcβ1,2. Di-6, which contains two sulfated peripheral branches, coelutes in region II with Di-1 and Di-2, both of which contain two sialylated peripheral branches (Fig. 7A). Similarly, desulfated Di-6, bearing two terminal β1,4-linked GalNAc residues, coelutes between regions III and IV with asialo Di-1 and asialo Di-2, both of which contain two terminal β1,4-linked Gal moieties (Fig. 7B).

Fig. 6. RCAII affinity HPLC of oligosaccharides bearing terminal β1,3- versus β1,4-linked Gal residues. N-Glycanase-released, reduced Di-8 (panel A), β-galactosidase- (Diplococcus) digested Di-8 (panel B), β-galactosidase- and β-N-acetylhexosaminidase- (Diplococcus) digested Di-8 (panel C), and Di-9 (panel D) oligosaccharides were analyzed by RCAII affinity HPLC. Bound oligosaccharides were eluted with 100 mM GalNAc and 1.0 N acetic acid in PBS/Na2SO4 at 30 min (arrow). Elution regions I-IV are indicated by brackets. Symbols for the schematically illustrated oligosaccharides are the same as in Fig. 1, with detailed structures shown in Table I.
is attached to the α1,3-linked core Man, reflects the type and linkage of the branch’s terminal moiety; i.e. interaction with β1,4-linked GalNAc (Fig. 7D and data not shown) > β1,4-linked Gal (Fig. 5C) > β1,3-linked Gal (Fig. 6C).

The affinity of RCAIII for dibranched oligosaccharides bearing terminal sulfate on the branch attached to the α1,3-linked core Man depends upon the composition of the branch attached to the α1,6-linked core Man. The sulfated/sialylated structure Di-5, which contains a typical sialylated branch attached to the α1,6-linked core Man, coelutes with Di-1, Di-2, and Di-6 in region II (Fig. 7E). Similarly, asialo Di-5 coelutes with asialo Di-1, asialo Di-2, and desulfated Di-6 between regions III and IV (Fig. 7F). Removal of the Gal residue from asialo Di-5, leaving only the β1,2-linked GlcNAc attached to the α1,6-linked core Man, results in elution of the oligosaccharide in region II (Fig. 7G). In contrast, Hyb-1 (Fig. 7C), bearing two Man residues attached to the α1,6-linked core Man, and asialo Di-5 treated with Diplococcal β-galactosidase and β-N-acetylgalactosaminidase to remove the peripheral branch attached to the α1,6-linked core Man (Fig. 7H), coelute in region I. Of note, Di-3, which is analogous to this latter form of Di-5, also elutes in region I (Fig. 5A).

Analysis of tri- and tetrabranched complex-type oligosaccharides by RCAIII affinity HPLC yielded more complicated separations than those obtained with dibranched structures. Whereas Di-1 and Di-2 are retarded but not bound by RCAIII, Tri-1 oligosaccharides bind to the lectin and are eluted in region IV (Fig. 8A). Removal of sialic acid from Tri-1 does not appear to significantly alter interaction with RCAIII, since asialo Tri-1 is eluted in region IV, slightly later than asialo Di-1 and asialo Di-2 (Fig. 8B). In contrast to the agalacto forms of Di-1 and Di-2, agalacto Tri-1 binds to RCAIII and is eluted in region III (Fig. 8C).

The presence of a peripheral branch attached to the C-6 position of the α1,6-linked core Man inhibits oligosaccharide interaction with RCAIII. Tetra-1, which is similar to Tri-1 except for the additional presence of a sialylated peripheral branch attached to the C-6 position of the α1,6-linked core Man, elutes in region I (Fig. 8F), whereas Tri-1 elutes in region IV (Fig. 8A). Like Tetra-1, Tri-2 bears a peripheral branch arising from the C-6 position of the α1,6-linked core Man. Asialo Tetra-1 (Fig. 8G) and Tri-2 (Fig. 8D), which contain 4 and 3 terminal β1,4-linked Gal residues, respectively, coelute in region III. Since the asialo forms of Di-1, Di-2 (Fig 4C), and Tri-1 (Fig 8B) elute slightly later than asialo Tetra-1 and Tri-2, the peripheral branch arising from the C-6 position of the α1,6-linked core Man must reduce oligosaccharide-RCAIII interaction, even in the presence of multiple β1,4-linked Gal residues. Agalacto Tri-2 (Fig. 8E) and agalacto Tetra-1 (Fig. 8H) coelute in region I, whereas agalacto Tri-1 (Fig. 8C) and agalacto Di-2 (Fig. 4D) elute in regions III and II, respectively. Thus, the presence of a peripheral branch containing a β1,6-linked GlcNAc attached to the α1,6-linked core Man diminishes the interaction of tri- and tetrabranched complex-type oligosaccharides with RCAIII both in the presence and absence of β1,4-linked Gal moieties.

The agalacto forms of Tri-1, Tri-2, and Tetra-1 were also digested with Diplococcal β-N-acetylgalactosaminidase, which selectively removes peripheral GlcNAc moieties (27). This enzyme releases both β1,2-linked GlcNAc residues from agalacto Tri-1, but only the β1,2-linked GlcNAc residue attached to the α1,3-linked core Man from agalacto Tri-2 and agalacto Tetra-1. The Diplococcal β-N-acetylgalactosaminidase-digested forms of agalacto Tri-1, agalacto Tri-2, and agalacto Tetra-1 all elute in region I during RCAIII affinity HPLC (data not shown). In the case of Tri-1, this indicates that 1 or both β1,2-linked GlcNAc residues as well as the β1,4-linked GlcNAc moiety contribute to the oligosaccharide’s interaction with RCAIII.

A number of other mono-, di-, and oligosaccharides were examined by RCAIII affinity HPLC. Oligosaccharide structures devoid of peripheral Gal, GlcNAc, GalNAc, and sialic acid, for example HM-1, elute in region I. Similarly, [3H]Gal, [3H]GlcNAc, [3H]UDP-Gal, and [3H]UDP-GalNAc all elute in region I. [3H]GalNAc is retarded by RCAIII, eluting in region II (11 min), as is [3H]lactosamine (Galβ1,4GlcNAc), which
also elutes in region II (15 min). The difference in interaction between UDP-GalNAc, which contains GalNAc in an α linkage, and free GalNAc or lactosamine suggests that the β anomer of GalNAc or Gal is essential for interaction with RCAI.

**DISCUSSION**

In this study, we have demonstrated that RCAI and RCAII affinity HPLC can be effectively utilized for the fractionation and characterization of N-glycanase-released, reduced oligosaccharides. Like RCAI and RCAII, we have found that leukoagglutinating phytohemagglutinin (L-PHA), erythroagglutinating phytohemagglutinin (E-PHA), *Datura stramonium* agglutinin, and *Vicia villosa* agglutinin display high degrees of specificity for oligosaccharides and that these lectins have a greater capacity to discriminate between closely related oligosaccharides than had previously been appreciated (7, 8). As a result, oligosaccharides elute at characteristic and reproducible positions during lectin affinity chromatography. Furthermore, our ability to detect subtle aspects of oligosaccharide-lectin interactions has been enhanced with lectin-silica affinity HPLC (7), as compared to lectin-agarose affinity chromatography. Thus, lectin affinity HPLC can be used for the separation of heterogeneous mixtures of oligosaccharides, for the characterization of oligosaccharide structures, and for the definition of lectin specificities. In addition, in this study we have found that silica-bound RCAI and RCAII are capable of discriminating between smaller structures, such as monoo- and disaccharides, which has not previously been possible with agarose-bound forms of these lectins. For example, lactosamine, reduced lactosamine, and reduced lactose are separated during RCAII affinity HPLC. Similarly, GalNAc and lactosamine are retarded during RCAII affinity HPLC, whereas GlcNAc and Gal are not.

Studies examining the oligosaccharide specificities of RCAI and RCAII have been performed in this (3) and other (1, 2, 4, 5) laboratories utilizing various techniques. Using $^{125}$I-labeled glycopeptides of known structure, we previously characterized the interaction of peptide-bound oligosaccharides with RCAI and RCAII using precipitation assays (3). Our current approach for analyzing RCAI and RCAII specificity differs significantly from that used previously. In the present study, we have analyzed N-glycanase-released, reduced oligosaccharides rather than Asn-linked structures and have examined oligosaccharide specificity by lectin affinity HPLC rather than by solution-binding assays. Nonetheless, in virtually all respects, the results presented in this study are in agreement with our previous conclusions about RCAI and RCAII specificities (3). This is true for weak as well as strong oligosaccharide-lectin interactions. For example, we previously found that the presence of α2,6-linked sialic acid decreases but does not abolish binding of oligosaccharides to RCAI. We now find that structures bearing α2,6-linked sialic acid are retarded during RCAII affinity HPLC, whereas those with α2,3-linked sialic acid are not. Although we have detected differences in the specificity of some lectins, such as L- and E-PHA (8), for Asn-linked versus N-glycanase-released oligosaccharides, this does not appear to be the case for either RCAI or RCAII.

If the elution position of an oligosaccharide during lectin affinity HPLC is considered indicative of the strength of its interaction with that lectin, then a number of new conclusions about the specificities of RCAI and RCAII can be made. Although the carbohydrate-binding β subunits of these lectins may be highly homologous (1-3), their specificities for mono-, di-, and oligosaccharides differ in several respects. Both lectins have a high affinity for lactosamine (Galβ1,4GlcNAc), consistent with their common ability to interact with complex-type oligosaccharides bearing one or more peripheral lactosamine moieties. The penultimate sugar residue is of major significance for interaction with RCAII, since reduced lactose and reduced lactosamine are less retarded during RCAII affinity HPLC than unreduced lactosamine, and Gal elutes unretarded. Similarly, oligosaccharides bound by RCAI can be eluted with lactose but not with Gal. In the case of RCAII, GalNAc interacts with the lectin almost as strongly as lactosamine, but free Gal does not interact. In addition, GalNAc but not Gal can be used to elute bound oligosaccharides bearing either terminal Gal or GalNAc from RCAII. These differences in RCAI and RCAII specificity can be utilized to distinguish larger structures containing terminal Galβ1,4GlcNAc from those bearing terminal Galβ1,4-GlcNAc: the former interact with RCAII but not RCAI, whereas the latter interact with both lectins. For oligosaccharides bearing both types of terminal sequences, analysis by *Vicia villosa* agglutinin affinity HPLC can be used to confirm the presence of terminal GalNAc, since this lectin does not interact with terminal Gal (7).

The interaction of oligosaccharides with RCAI and RCAII is influenced by the location and number of peripheral branches bearing Galβ1,4GlcNAc as well as by other structural features of the peripheral branches themselves. For example, RCAI and RCAII display different specificities for oligosaccharides bearing α2,3- versus α2,6-linked sialic acid moieties. The presence of α2,3-linked sialic acid abolishes interaction of dibranched complex-type oligosaccharides with RCAI, whereas the presence of α2,6-linked sialic acid on such structures reduces but does not eliminate lectin interaction. In contrast, the presence of either α2,3- or α2,6-linked sialic acid on dibranched complex-type oligosaccharides reduces but does not eliminate interaction with RCAII. As discussed above, these results are in agreement with our previous analyses using soluble lectins and precipitation assays (3). Of note, the presence of α2,3-linked sialic acid markedly enhances interaction with L- and E-PHA, whereas the presence of α2,6-linked sialic acid abolishes interaction with these lectins (8).

RCAI and RCAII display opposite preferences for the location of peripheral lactosamine-containing branches (Figs. 1 and 5). Therefore, lectin affinity HPLC with RCAI and RCAII can be used to determine the attachment site (α1,3- or α1,6-linked core Man) of a peripheral branch. RCAI and RCAII can also be used to distinguish between terminal β1,3- versus β1,4-linked Gal moieties. For example, RCAI displays little affinity for oligosaccharides bearing either 1 or 2 terminal β1,3-linked Gal residues, whereas RCAII binds such structures, requiring GalNAc for elution. Both lectins strongly interact with oligosaccharides bearing terminal β1,4-linked Gal residues.

The desialylated forms of tri- and tetra- branched complex-type oligosaccharides are bound by RCAII. Subsequent removal of Gal abolishes interaction of these structures with RCAII. In contrast, RCAII discriminates between various agalacto tri- and tetrabranched oligosaccharides, depending upon the attachment sites of their peripheral GlcNAc residues. Tribranched oligosaccharides devoid of sialic acid and Gal continue to interact with RCAII as long as there is not a β1,6-linked GlcNAc residue attached to the α1,6-linked core Man. The presence of such a GlcNAc residue abolishes interaction of agalacto oligosaccharides with RCAII. Therefore, RCAII does not require peripheral Gal or GalNAc for interaction with certain complex-type oligosaccharides. Even in the presence of terminal Gal, tribranched oligosaccharides with a peripheral branch attached to the C-6 position of the α1,6-linked core Man (asialo Tr-II) do not interact as strongly.
with RCA₂ as tribranched structures without this peripheral branch (asialo Tri-1). Thus, the presence of a substituent attached to the C-6 position of the α1,6-linked core Man has a detrimental effect on interaction with RCA₃.

In addition to providing a useful method for fractionating and analyzing oligosaccharide structures, our studies using lectin affinity HPLC have further demonstrated the exquisite specificity displayed by lectins for oligosaccharide structural features. Such a high degree of specificity could be essential for lectins in higher organisms, which may play a role in binding and transport of soluble glycoconjugates and/or in cell-cell recognition. The ability of plant lectins, such as RCA₁, to bind and transport of soluble glycoconjugates and/or in cell-cell recognition. The ability of plant lectins, such as RCA₁, RCA₂, L-PHA, E-PHA, Datura stramonium agglutinin, and Vicia villosa agglutinin (7, 8), to distinguish among a large number of closely related oligosaccharides emphasizes the importance of considering lectin specificity within the context of oligosaccharide structures as well as competitive mono- and disaccharide haptenes. Our observations also indicate that without detailed knowledge of the carbohydrate structures under study, one must interpret the binding of lectins to intact glycoproteins and cell surfaces with considerable caution.

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REFERENCES

1. Olsnes, S., and Pihl, A. (1982) in The Molecular Actions of Toxins and Viruses (Cohen, S., and van Heyningen, S., eds) pp. 5-105, Elsevier Scientific Publishing, Amsterdam.
2. Goldstein, I. J., and Poretz, R. D. (1986) in The Lectins: Properties, Functions, and Applications in Biology and Medicine (Lien- ner, I. E., Sharon, N., and Goldstein, I. J., eds) pp. 33-247, Academic Press, Orlando, FL.
3. Baenziger, J. U., and Fiete, D. (1979) J. Biol. Chem. 254, 9795-9799.
4. Merkle, R. K., and Cummings, R. D. (1986) Methods Enzymol. 138, 232-259.
5. Debray, H., Decout, D., Strecker, G., Spik, G., and Montreuil, J. (1981) Eur. J. Biochem. 117, 41-55.
6. Narasimhan, S., Freed, J. C., and Schachter, H. (1986) Carbohydr. Res. 149, 65-83.
7. Green, E. D., Brodbeck, R. M., and Baenziger, J. U. (1987) Anal. Biochem., in press.
8. Green, E. D., and Baenziger, J. U. (1987) J. Biol. Chem. 262, 12018-12029.
9. Spik, G., Bayard, B., Fournet, B., Strecker, G., Bouquelet, S., and Montreuil, J. (1975) FEBS Lett. 50, 296-299.
10. Endo, Y., Yamashita, K., Tachibana, Y., Tojo, S., and Kobata, A. (1979) J. Biochem. (Tokyo) 85, 669-679.
11. Mizuochi, T., and Kobata, A. (1980) Biochem. Biophys. Res. Commun. 97, 772-778.
12. Kessler, M. J., Reddy, M. S., Shah, R. H., and Bahl, O. P. (1979) J. Biol. Chem. 254, 7901-7908.
13. Tai, T., Ito, S., Yamashita, K., Muramatsu, T., and Kobata, A. (1975) Biochem. Biophys. Res. Commun. 65, 968-974.
14. Green, E. D., Baenziger, J. U., and Boime, I. (1985) J. Biol. Chem. 260, 15631-15638.
15. Green, E. D., van Halbeek, H., Boime, I., and Baenziger, J. U. (1985) J. Biol. Chem. 260, 15623-15630.
16. Green, E. D., Boime, I., and Baenziger, J. U. (1986) J. Biol. Chem. 261, 16309-16316.
17. Mizuochi, T., Yamashita, K., Fujikama, K., Titan, K., and Kobata, A. (1980) J. Biol. Chem. 255, 3526-3531.
18. Nilsson, B., Norden, N. E., and Svensson, S. (1979) J. Biol. Chem. 254, 4545-4553.
19. Cummings, R. D., and Kornfeld, S. (1984) J. Biol. Chem. 259, 6253-6260.
20. Fournet, B., Montreuil, J., Strecker, G., Dorland, L., Haverkamp, J., Vliegenthart, J. F. G., Binette, J. P., and Schmid, K. (1978) Biochemistry 17, 5206-5214.
21. Chapman, A., and Kornfeld, R. (1975) J. Biol. Chem. 254, 816-823.
22. Chapman, A., and Kornfeld, R. (1975) J. Biol. Chem. 254, 824-828.
23. Green, E. D., and Baenziger, J. U. (1986) Anal. Biochem. 158, 42-49.
24. Paulson, J. C., Sadler, J. E., and Hill, R. L. (1979) J. Biol. Chem. 254, 2120-2124.
25. Paulson, J. C., Weinstein, J., Dorland, L., van Halbeek, H., and Vliegenthart, J. F. G. (1982) J. Biol. Chem. 257, 12734-12738.
26. Paulson, J. C., Priels, J. P., Glasgow, L. R., and Hill, R. L. (1978) J. Biol. Chem. 253, 5617-5624.
27. Yamashita, K., Ohkura, T., Yoshima, H., and Kobata, A. (1981) Biochem. Biophys. Res. Commun. 100, 226-232.