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THE EFFECT OF A "BISECTING" *N*-ACETYLGLUCOSAMINYL GROUP ON THE BINDING OF BIANTENNARY, COMPLEX OLIGOSACCHARIDES TO CONCANAVALIN A, *Phaseolus vulgaris* ERYTHROAGGLUTININ (E-PHA), AND *Ricinus communis* AGGLUTININ (RCA-120) IMMOBILIZED ON AGAROSE*

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ABSTRACT

The effect of a "bisecting" 2-acetamido-2-deoxy-β-D-glucopyranosyl group, linked (1→4) to the β-D-mannopyranosyl group of asparagine-linked complex and hybrid oligosaccharides, on the binding of [^{14}C]acetylated glycopeptides to columns of immobilized concanavalin A (Con A), *Phaseolus vulgaris* erythroagglutinin (E-PHA), and *Ricinus communis* agglutinin-120 (RCA-120) was investigated. The presence of this "bisecting" GlcNAc group caused significant inhibition of the binding to Con A-agarose of biantennary complex glycopeptides in which the two branches are terminated at their nonreducing ends by two GlcNAc groups, or by a Gal and a GlcNAc group, or by two Gal groups, or by a Man and a GlcNAc group. Binding of biantennary, complex glycopeptides to E-PHA–agarose required a "bisecting" GlcNAc group, a Gal group at the nonreducing terminus of the α-D-Man-*p*-(1→6) branch, and a terminal or internal GlcNAc residue linked β-(1→2) to the α-D-Man*p*-(1→3) branch. Binding to RCA-120–agarose occurred only when at least one nonreducing terminal Gal group was present, and increased as the proportion of terminal Gal groups increased; the presence of a "bisecting" GlcNAc group caused either enhancement or inhibition of these binding patterns. It is concluded that a "bisecting" GlcNAc group affects the binding of glycopeptides to all three lectin columns.

INTRODUCTION

Complex Asn-linked oligosaccharides consist of a $(Man)_3(GlcNAc)_2Asn$ core to which are attached two or more branches. The assembly of branches occurs in a

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highly ordered manner[1]. The insertion of a "bisecting" β-D-GlcNAc group, linked (1→4) to the β-D-Man residue of the trimannosyl core, during this process[2] prevents the action of the following four enzymes: (i) D-GlcNAc-transferase II which initiates the branch attached β-D-(1→2) to the α-D-Man-(1→6) residue of the core[3], (ii) D-GlcNAc-transferase IV which initiates the branch attached β-(1→4) to the α-D-Man-(1→3) residue of the core[4], (iii) the (1→6)-α-L-Fuc-transferase which inserts an L-Fuc group into the Asn-linked GlcNAc residue of the core[5], and (iv) α-D-mannosidase II which removes the last two Man residues during the processing of high-mannose to complex oligosaccharides[6].

The inhibitory action of the "bisecting" GlcNAc group is believed to be due to steric obstruction of the region **1** of the substrate, and it has been suggested that this region is a binding site for all of the aforementioned four enzymes[7]. We have recently reported[8] the inhibitory effect of a "bisecting" GlcNAc group on the galactosylation by bovine milk UDP-Gal:GlcNAc (1→4)-β-D-galactosyltransferase of a complex, biantennary glycopeptide having two GlcNAc groups at the nonreducing termini. Steric obstruction of the region **1** of the substrate is also believed to be responsible for this effect.

It has been observed that a "bisecting" GlcNAc group reduces the binding of glycopeptides to Con A–agarose[2,4,9]. We have therefore undertaken a systematic study of the behavior of some "bisected" and "nonbisected" glycopeptides on columns of immobilized lectins concanavalin A (Con A), *Phaseolus vulgaris* erythroagglutinin (E-PHA), and *Ricinus communis* agglutinin-120 (RCA-120). These results confirm and extend previous studies on Con A–agarose[2,9–14], E-PHA–agarose[15–18], and RCA-120–agarose[19].

β-D-Glc*p*NAc-(1→2)-α-D-Man*p*-(1→3)-β-D-Man*p*-(1→4)-

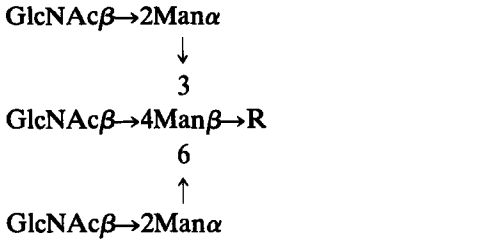
1

Galβ→4GlcNAcβ→2Manα→3Manβ→R
6
↑
Galβ→4GlcNAcβ→2Manα

2* R = X
3 R = Y

GlcNAcβ→2Manα→3Manβ→R
6
↑
GlcNAcβ→2Manα

4 R = X



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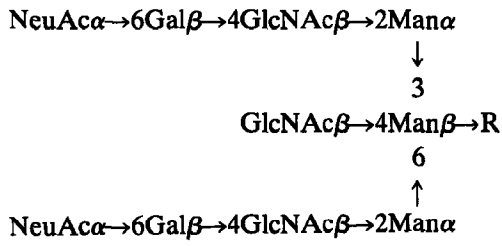

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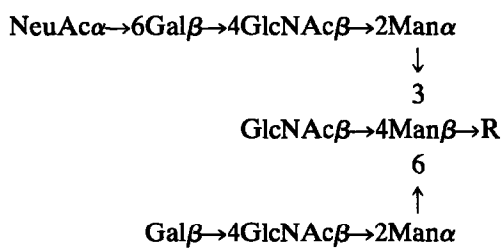

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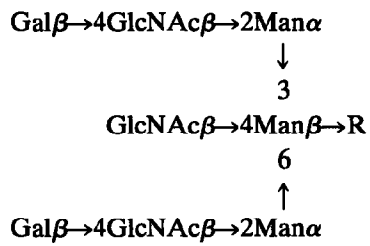

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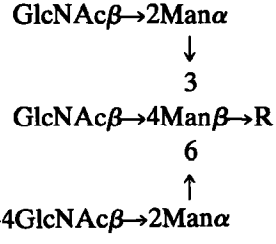

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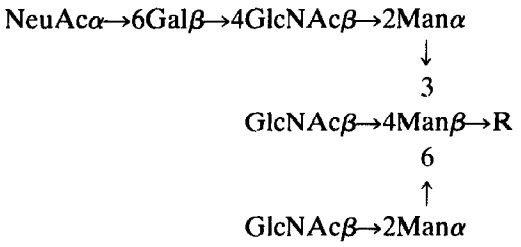

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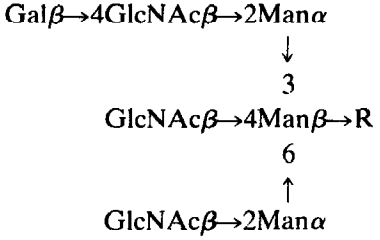

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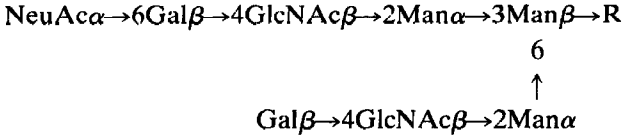

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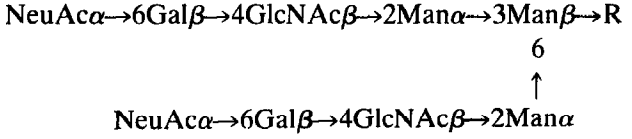

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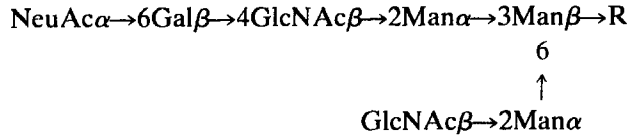

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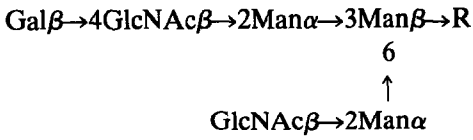

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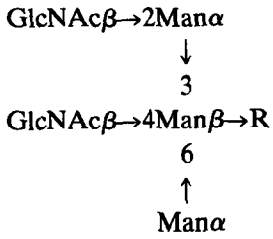

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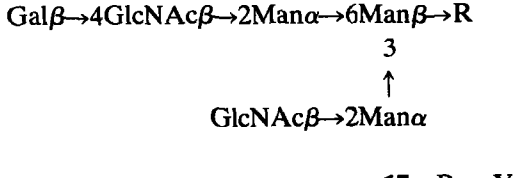

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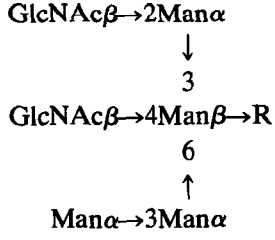

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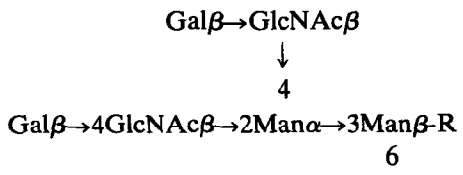

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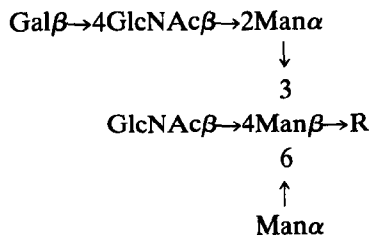

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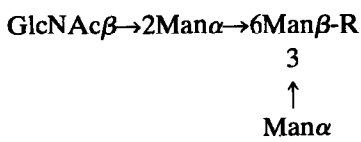

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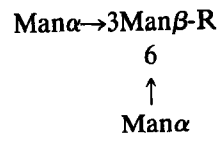


22 R = Y

GlcNAcβ→2Manα→3Manβ-R
6
↑
Manα

23 R = Y

Galβ→4GlcNAcβ→2Manα→3Manβ-R
6
↑
Manα

24 R = Y

NeuAcα→6Galβ→4GlcNAcβ→2Manα→3Manβ-R
6
↑
Manα

25 R = Y

Galβ→4GlcNAcβ→2Manα→6Manβ-R
3
↑
Manα

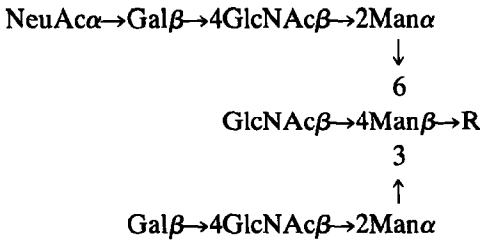
26 R = Y

NeuAcα→Galβ→4GlcNAcβ
↓
4
NeuAcα→Galβ→4GlcNAcβ→2Manα→3Manβ-R
6
↑
NeuAcα→Galβ→4GlcNAcβ→2Manα

27 R = Y

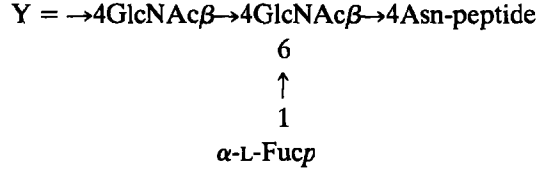
GlcNAcβ
↓
4
GlcNAcβ→2Manα→3Manβ-R
6
↑
GlcNAcβ→2Manα

28 R = X
29 R = Y



30 R = Y

X = →4GlcNAcβ→4GlcNAcβ→4Asn-peptide



*In abbreviated structures, the D configuration, pyranose form, and linkage at C-1 of Gal, GlcNAc, and Man, and the pyranose form and linkage at C-2 of NeuAc are assumed.

EXPERIMENTAL

Materials. — [1-^{14}C]Acetic anhydride (10 mCi/mmol) was from New England Nuclear. Jack bean β-D-galactosidase (19 units/mg of protein) and Tris [2-amino-2-(hydroxymethyl)-1,3-propanediol] were from Sigma. A *Clostridium perfringens* culture supernatant was used to prepare[20] a mixture of β-D-galactosidase (1.5 units/mL) and *N*-acetyl-β-D-glucosaminidase (2 units/mL). One enzyme unit is defined as causing the conversion of 1 μmol of substrate/min. Con A–Sepharose 4B (lot No.HC24782, 10 mg of protein/mL of gel) was from Pharmacia, E-PHA–agarose (lot No.0201F, 5 mg of protein/mL of gel) was from EY Labs, and RCA-120–agarose (lot No.AR 27, 1.2 mg of protein/mL of gel) was from Miles–Yeda. Pronase and human fibrinogen (lot No.102009) were from Calbiochem–Behring. Methyl α-D-glucopyranoside was from Nutritional Biochemical Corp. or Aldrich Chem. Corp. D-mannose from Pfanstiehl Labs. and lactose from BDH. Bio-Gel P-2 (100–200 and 200–400 mesh) and P-4 (>400 mesh, extra fine) were from Bio-Rad Labs. Deuterium oxide was from Aldrich Chem. Corp. (99.8 and 100%) or Merck Sharpe & Dohme (99.7 and 100%). Aqueous counting scintillant

and organic counting scintillant solutions were from Amersham. All other chemicals were reagent grade and used without further treatment

Preparation of glycopeptides. — Glycopeptide **2** was prepared from a Pronase digest of human asialofibrinogen[4,8] and digested with jack bean β-D-galactosidase[4,8] to give glycopeptide **4** which was purified from this digest by gel filtration on Bio-Gel P-2 and preparative, high-voltage paper electrophoresis[4,8,21] in 1% $Na_2B_4O_7$.

The "bisected" glycopeptide **5** was prepared from hen ovalbumin[2,5] (Fraction V-1) and purified by passage through a Bio-Gel P-4 column[8]. The "bisected" glycopeptides **6, 7, 8** and **9**, all containing core L-fucose residues, were kind gifts of Dr. J. P. Carver, and were prepared from a Pronase digest of IgG (Hom) obtained from a human multiple-myeloma patient, as previously described[22,23]. Glycopeptides **6** and **7** were from the light chains, and **8** and **9** from the heavy chains. The neutral glycopeptides were purified by preparative Con A–Sepharose chromatography, **8** being unbound on this column and **9** being retarded.

Glycopeptide **7** from IgG (Hom) was acetylated with [^{14}C]acetic anhydride and purified[10,21]. Glycopeptide **10** was prepared by removal of galactose from **7** with β-D-galactosidase and was purified by gel filtration on a Bio-Gel P-2 column (100–200 mesh, 1.2 × 48 cm) in water[8]; it was hydrolyzed with 2M acetic acid at 100° for 15 min to remove sialic acid[24]. The resulting glycopeptide **11** was purified on a Bio-Gel P-2 column (100–200 mesh, 1.2 × 48 cm) in water[8]. As a control experiment to ensure that our conditions of mild acid hydrolysis did not remove the "bisecting" GlcNAc group, **6** was hydrolyzed to give **8** under similar conditions. The conversion of **7** into **10**, of **10** into **11**, and of **6** into **8** were monitored by high-voltage paper electrophoresis[21] in 1% $Na_2B_4O_7$, and by lectin column chromatography, the radioactivity being used to detect the positions of the various glycopeptides.

Glycopeptides **12** and **13** were prepared from Pronase digests of IgG from a human multiple-myeloma patient as previously described[10,21,25]. Glycopeptide **12** was degraded into **14** by treatment with β-D-galactosidase and **14** was hydrolyzed with 50mM HCl at 80° for 1 h to remove sialic acid to form[8] **15**, which was purified by preparative high-voltage paper electrophoresis in 1% $Na_2B_4O_7$.

Glycopeptides **3** (ref. 10), **16** (ref. 21), and **17** (ref. 25), containing core L-fucose residues, were prepared from a Pronase digest of human IgG (Tem). Ovalbumin glycopeptide III-A (**18**) was prepared from a Pronase digest of hen ovalbumin[21]. Glycopeptide **19** from human α_1-acid glycoprotein was a kind gift from Dr. K. Schmid.

All the aforementioned glycopeptides, except **10, 11**, and **19**, were identified[21,25,26] by their characteristic ^{1}H-n.m.r. spectra at 360 MHz. The ^{1}H-n.m.r. spectra indicated the purity of these glycopeptide preparations to be at least 70%. Not enough **10, 11,** or **19** were available for ^{1}H-n.m.r. spectroscopy; however, lectin-column chromatography showed the purity of these preparations to be 75, 58, and 57%, respectively. The structures of the contaminants in our glycopeptide preparations were not determined. However, the nature of the major component in all our

preparations has been established and all conclusions in this paper are based only on these major components.

Galactosylation of ovalbumin glycopeptide **18** and of IgG glycopeptide **16** (to form **20**) was carried out with bovine milk UDP-Gal:GlcNAc (1→4)-β-D-galactosyltransferase as previously described[8].

Con A–Sepharose chromatography. — Conditions were modified from those described by Narasimhan *et al.*[10]. Columns (0.7 × 9 cm) were eluted at room temperature at 10 mL/h, with 10mM Tris · HCl (pH 7.5), 0.1M NaCl (30–80 mL depending on the sample), followed by 10mM methyl α-D-glucopyranoside in the same buffer (30–40 mL), and finally with 0.1M methyl α-D-glucopyranoside in the same buffer (20–50 mL). Fraction size was 1 mL and the radioactivity of the entire fraction was counted. Con A–Sepharose could be used for ~20 experiments without obvious deterioration and could be stored for months at 4° in buffer containing 0.02% NaN_3. However, repeated use of Con A–Sepharose caused gradual loss of Con A due to bleeding. Certain glycopeptides, such as **2**, adhere weakly to fresh Con A–Sepharose and could be eluted with 10mM methyl α-D-glucopyranoside; they will not, however, adhere to Con A–Sepharose that has been used more than 20 times but will be eluted in a retarded manner from such a column with buffer lacking methyl α-D-glucopyranoside.

E-PHA–agarose chromatography. — Conditions were modified slightly from those of Cummings and Kornfeld[15] in that the column size (0.5 × 60 cm) was doubled. Columns were eluted at room temperature at 2.6 mL/h with phosphate-buffered saline solution (6.7mM KH_2PO_4, pH 7.4, 0.15M NaCl) containing 0.02% NaN_3. Fraction size was 1 mL and the radioactivity of the entire fraction was counted. E-PHA–agarose was used repeatedly for 6 months without apparent deterioration.

RCA-120–agarose chromatography. — Conditions were modified from those of Gleeson and Schachter[4]. Column size was 0.5 × 44 cm and elutions were performed at room temperature at 4.2 mL/h in 10mM Tris · HCl (pH 7.5), 0.1M NaCl, and 0.02% NaN_3. The samples were allowed to interact with the gel for 1 h before elution. The column was eluted with the aforementioned buffer (90 mL) and then with 0.1M lactose in the same buffer (20 mL). Fraction size was 1 mL and the radioactivity of the entire fraction was counted. RCA-120–agarose was used repeatedly for 6 months without apparent deterioration.

Other methods. — Glycopeptides were *N*-[^{14}C]acetylated with [1-^{14}C]acetic anhydride and acetylated glycopeptides were purified as previously described[10,21]. Glycopeptide concentrations were determined by amino acid analysis for aspartic acid as described previously[21]. Radioactivity on paper was counted in OCS, and that of aqueous solutions in 10 mL ACS with an LKB Rackbeta counter. ^{1}H-N.m.r. spectroscopy was performed, as previously described[21], on solutions of samples that had been exchanged in 100% D_2O and by use of a Nicolet 360 MHz spectrometer[26]. Glycopeptides were monitored in column effluents either by the phenol–H_2SO_4 method[27] with D-mannose as a standard or by radioactivity counting. Sialic acid

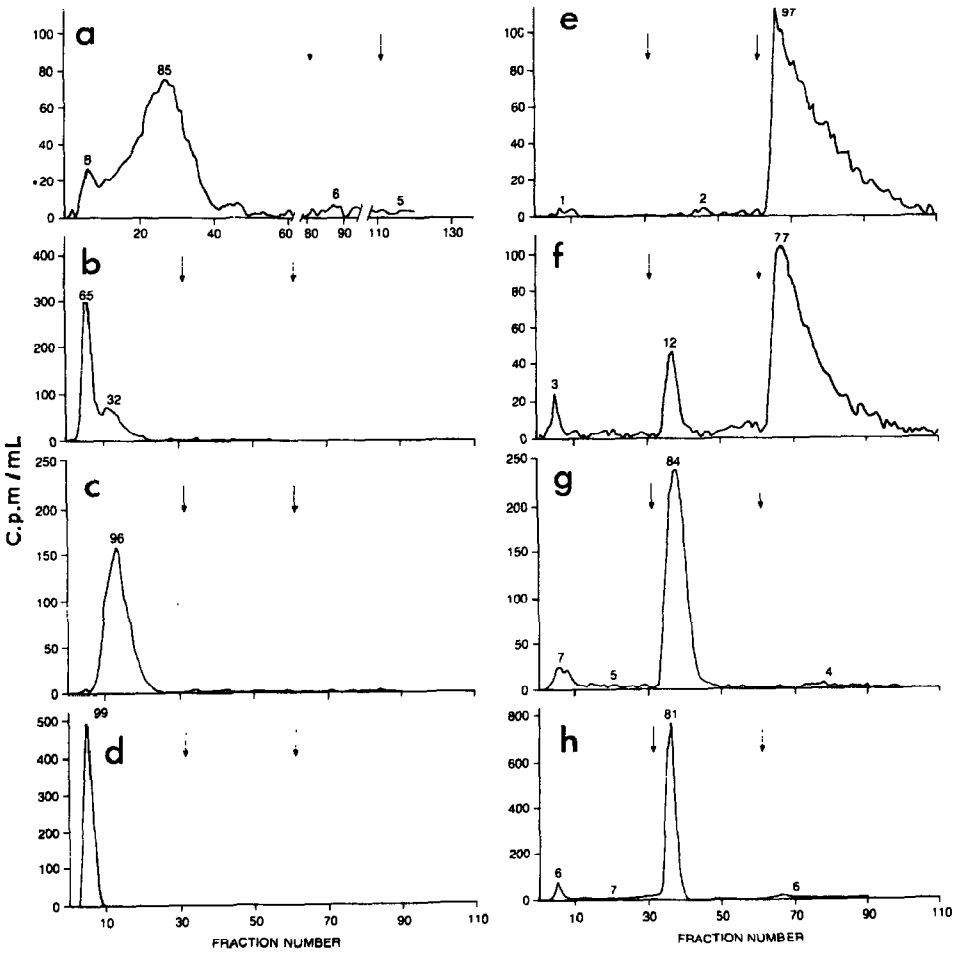


Fig. 1. Elution profiles of standard *N*-[^{14}C]acetylated glycopeptides on Con A–Sepharose columns, as described in the Experimental section. The two arrows indicate the points of addition of 10mM and 100mM methyl α-D-glucopyranoside, respectively. The void volume is at 5 mL. The numbers near the peaks represent the percent of radioactivity recovered in the peak. The radioactivity loaded per column varied between 1260 and 2750 c.p.m., and the percent of recovery between 86 and 100%: (a) **5**, (b) **11**, (c) **9**, (d) **8**, (e) **4**, (f) **15**, (g) **17**, and (h) **3**.

release was monitored by the method of Warren[28], galactose release by the method of Finch *et al.*[29], and 2-acetamido-2-deoxyglucose release by the method of Reissig *et al.*[30].

RESULTS

Con A–Sepharose chromatography. — Typical elution profiles on Con A–Sepharose of standard *N*-[^{14}C]acetylated glycopeptides are shown in Fig. 1. All

TABLE I

PATTERNS OF COLUMN CHROMATOGRAPHY ON LECTIN–AGAROSE

Structure	*"Non-bisected" glycopeptides*			*Structure*	*"Bisected" glycopeptides*		
	Con A[a]	*E-PHA*[b]	*RCA-120*[c]		*Con A*[a]	*E-PHA*[b]	*RCA-120*[c]
13	B–C[d]	NR[d]	9[e]	**6**		12	11[f]
12	B–C	NR[d]	20	**7**		40	60[f]
3	B–C	15	50	**8**	A[f]	40	60
17	B–C	15[f]	20[f]	**9**	B[f]	40	29[f]
15	E	15[f]	30	**11**	A[f]	17	13[f]
4	E[g]	12[e,f]	9[e]	**5**	B[e]	16[e]	9[e]
14	E	12[f]		**10**		17[f]	9[f]
25	D						
24	D			**20**		12[f]	13[f]
23	D			**16**	B	12[f]	
22	D		NR[d]				
				30[h]		NR[d]	
26	C[f]						
				26		NR[d]	
27	A[d]	NR[d]					
19		15[e]	68[e]				
				19[h]		R[d]	
28	A[e]						
				28[h]	A[e]		
29	A						

[a](A) No retention, (B) was retarded but eluted with buffer alone, (C) bound to column and could be eluted with 10mM methyl α-D-glucopyranoside, (D) bound and was eluted as a sharp peak with 0.1M methyl α-D-glucopyranoside, and (E) bound and was eluted as a broad peak with 0.1M methyl α-D-glucopyranoside. [b]Elution is given as mL of eluent under the conditions described herein or as NR (not retained) and R (retained). [c]Same as for E-PHA–agarose. [d]These structures have not been studied in our laboratory and have been reported by others, as described in the text. [e]Devoid of (1→6)-linked α-L-fucopyranosyl group in the care. [f]These patterns have, to our knowledge, not been previously published. [g]Same result was obtained with or without a (1→6)-linked α-L-fucopyranosyl group in the care. [h]With "bisecting" β-D-GlcNAc*p*-(1→4) group.

the "nonbisected" biantennary glycopeptides tested bound to Con A–Sepharose (Fig. 1 and Table I). Glycopeptides having a terminal GlcNAc group on the α-D-Man-(1→6) branch, such as **4** (Fig. 1e), **14** (Table I), and **15** (Fig. 1f), bind strongly and are eluted as a broad peak with 0.1M methyl α-D-glucopyranoside; **21** should also show this pattern but has not been tested. Previous work from several laboratories[9–14] has shown that glycopeptides having a terminal Man group on the α-D-Man-(1→6) branch (**22, 23, 24,** and **25**; Table I) also bind strongly to Con A–Sepharose but are eluted as a sharp peak with 0.1M methyl α-D-glucopyranoside. "Nonbisected" biantennary glycopeptides having an α-Man-(1→6) branch ending with a terminal Gal group (**17**, Fig. 1g; **3**, Fig. 1h; **12**; and **26**, Table I) or a sialic

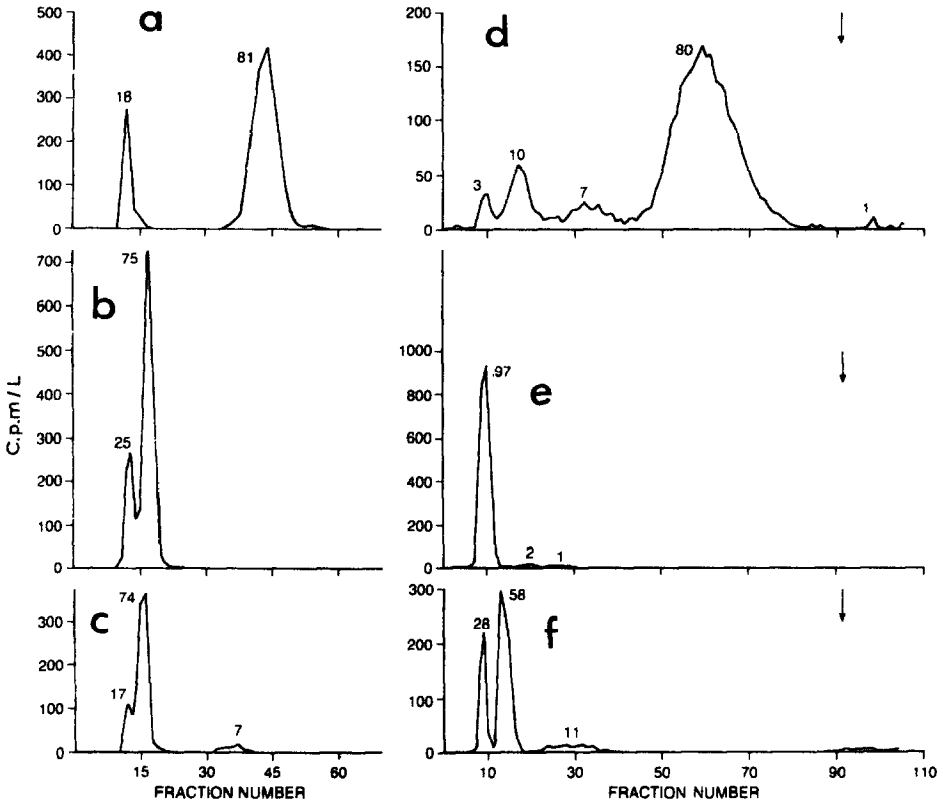


Fig. 2. Elution profiles on E-PHA–agarose (a–c) and RCA-120–agarose (d–f) columns, as described in the Experimental section, showing the results of β-D-galactosidase digestion of **7** to form **10**, and of sialic acid removal from **10** to form **11**. The arrows in (d) to (f) indicate the point of addition of 0.1M lactose. The void volume is at 12.0 mL in (a), (b), and (c); and at 9.0 mL in (d), (e), and (f). The numbers near the peaks represent the percent of radioactivity recovered in the peak. The radioactivity loaded on every column varied between 1470 and 3260 c.p.m., and the percent of recovery between 84 and 100%: (a,d) **7**; (b,e) **10**; and (c,f) **11**.

acid group (**13**, Table I) bind weakly to the column and require only 0.01M methyl α-D-glucopyranoside for elution. This suggests that a terminal GlcNAc group on the α-D-Man*p*-(1→6) branch favors very tight binding.

It is to be noted that glycopeptide **24** binds tightly to Con A–Sepharose, whereas glycopeptide **26** binds weakly (Table I). This observation indicates that the α-D-Man*p*-(1→6) branch plays a more decisive role than the α-D-Man*p*-(1→3) branch in controlling interaction with Con A–Sepharose.

The "bisected" derivatives of five "nonbisected" glycopeptides that adhere to Con A–Sepharose have been tested [**5**, **8**, **9**, and **11**, Fig. 1a–d; **16**, Table I]. In all cases, the presence of a "bisecting" GlcNAc group weakens the binding to Con A–Sepharose. Glycopeptides **8** (Fig. 1d) and **11** (Fig. 1b) did not interact with the column and were eluted in the void volume. Glycopeptides **5** (Fig. 1a), **9** (Fig. 1c),

and **16** (Table I) showed a retarded elution in the absence of methyl α-D-glucopyranoside in the buffer.

Preparation of glycopeptides **10** *and* **11**. — These glycopeptides were derived by sequential degradation of **7**, the latter being characterized by ^{1}H-n.m.r. spectroscopy. These glycopeptides were obtained in amounts insufficient for proper ^{1}H-n.m.r. analysis, and identification was based on high-voltage, paper electrophoresis in 1% borate (data not shown) and on lectin-affinity chromatography (Fig. 2). Glycopeptide **7** interacted strongly with both E-PHA–agarose (Fig. 2a) and RCA-120–agarose (Fig. 2d). Glycopeptide **10**, the product of β-D-galactosidase treatment of **7**, showed a greatly decreased interaction with both lectin columns (Fig. 2b,e). Mild acid hydrolysis was used to remove sialic acid from **10** to form **11**. Both **10** and **11** interacted weakly with E-PHA–agarose (Fig. 2b,c). However, whereas **10** was eluted in the void volume of RCA-120–agarose, **11** interacted weakly with this lectin (Fig. 2f) due to the exposure of a terminal Gal group.

We have found that some conditions of mild acid hydrolysis remove the "bisecting" GlcNAc group[5]. To ensure that the conditions of mild acid hydrolysis used to convert **10** into **11** did not remove the "bisecting" GlcNAc group, control experiments were carried out in which **6** was subjected to mild acid hydrolysis and the product analyzed on E-PHA–agarose. Glycopeptide **6** passed through the lectin

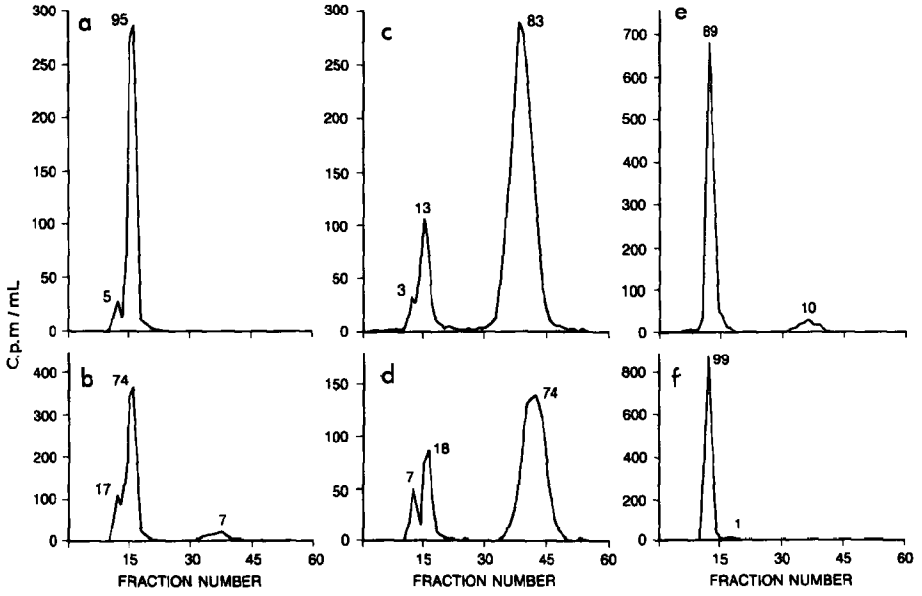


Fig. 3. Elution profiles of standard *N*-[^{14}C]acetylated glycopeptides on E-PHA–agarose columns, as described in the Experimental section. The void volume is at 12.0 mL. The numbers near the peaks represent the percent of radioactivity recovered in the peak. The radioactivity loaded per column varied between 1060 and 2520 c.p.m., and the percent of recovery between 75 and 95%: (a) **5**, (b) **11**, (c) **9**, (d) **8**, (e) **6**, and (f) **16**.

column unretarded (Fig. 3e), as previously described by others[15,18]. The product of mild acid hydrolysis of **6** was eluted at 40 mL (data not shown), in a position identical with that of a standard of **8** (Fig. 3d). Since the "bisecting" GlcNAc group is essential for strong interaction with E-PHA–agarose[15–18], it can be concluded that this group was not removed by the hydrolysis procedure.

Further confirmation of the conversion of **6** into **8** by mild acid hydrolysis was obtained by the behavior of these glycopeptides on RCA-120–agarose, *i.e.*, **6** passed through the column very slightly retarded, whereas the asialo derivative **8** was strongly retarded (Table I).

E-PHA–agarose chromatography. — Fig. 3 and Table I summarize our findings with E-PHA–agarose. Strong interaction with the lectin required both a "bisecting" GlcNAc group and a terminal, nonreducing Gal group on the α-D-Man*p*-(1→6) branch (**7**, Fig. 2a; **8**, Fig. 3d; and **9**, Fig. 3c), as previously found by others[15–18]. "Bisected" glycopeptides having a terminal Gal group only on the α-D-Man*p*-(1→3) branch (**11**, Fig. 3b; and **20**, Table I) interacted weakly or not at all with the lectin column. It is of interest that "bisected" glycopeptides having a terminal GlcNAc group on the α-D-Man*p*-(1→6) branch (**5**, Fig. 3a; **11**, Fig. 3b; and **10**, Table I) all interacted weakly with the lectin column, and that removal of this GlcNAc group (**16**, Fig. 3f; **20**, Table I) led to complete loss of interaction. Therefore, this terminal GlcNAc group must interact weakly with the lectin; this was not observed in previous studies by others[17,18], probably because shorter E-PHA–agarose columns were used. "Bisected" glycopeptides **6** (Fig. 3c), **18**, and **18** galactosylated on its α-D-Man*p*-(1→3) branch (data not shown) all passed through the column in the void volume, indicating the importance of a terminal Gal group on the α-D-Man*p*-(1→6) branch.

Table I indicates the behavior on E-PHA–agarose of several "nonbisected" glycopeptides not previously studied. It is seen that "nonbisected" compounds having terminal Gal groups (**3, 15, 17**, and **19**) all interacted weakly with E-PHA–agarose, but that no interaction in the absence of a terminal Gal group (**3** and **13**) was observed.

RCA-120–agarose chromatography. — Fig. 4 and Table I summarize the profiles obtained with RCA-120–agarose. No interaction was observed in the absence of a terminal Gal group [**13, 4** (Fig. 4e), **5** (Fig. 4a), and **10** (Fig. 2e)]. A single terminal Gal group caused interaction whether it is on the α-D-Man*p*-(1→3) or α-D-Man*p*-(1→6) branch, both in the absence and presence of a "bisecting" GlcNAc group [**7** (Fig. 2d), **9** (Fig. 4c), **11** (Fig. 4b), **12, 15** (Fig. 4f), **17** (Fig. 4g), and **20**; galactosylated ovalbumin glycopeptide III-A was eluted at 21 mL]. The "bisecting" GlcNAc group caused minor enhancement of binding of **17** and minor inhibition of binding of **15**. Two terminal Gal groups resulted in even stronger interaction [**3** (Fig. 4h) and **8** (Fig. 4d)]; a "bisecting" GlcNAc group enhanced this binding slightly. The tightest binding to RCA-120–agarose was observed with three terminal Gal groups (**19**, Fig. 4i).

Association constants have been reported for the binding of "nonbisected"

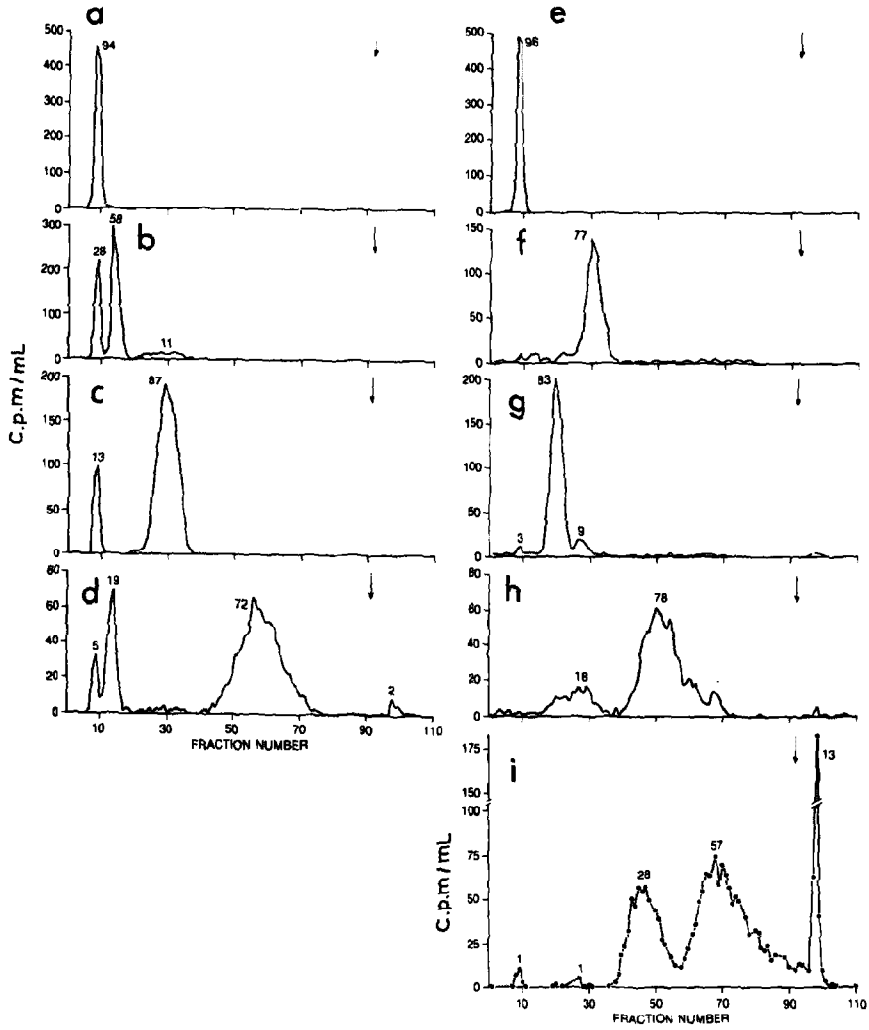


Fig. 4. Elution profiles of standard *N*-[^{14}C]acetylated glycopeptides on RCA-120–agarose columns, as described in the Experimental section. The arrow indicates the point of addition of 0.1M lactose. The void volume is at 9.0 mL. The numbers near the peaks represent the percent of radioactivity recovered in the peak. The radioactivity loaded on every column varied between 1060 and 2690 c.p.m., and the percent of recovery between 86 and 100%: (a) **5**, (b) **11**, (c) **9**, (d) **8**, (e) **4**, (f) **15**, (g) **17**, (h) **3**, and (i) **19**.

glycopeptides to RCA-120–agarose[32]. These are in general agreement with the results summarized in Table I. However, the interactions that we observed with both **12** and **13** were lower than those predicted from the association constants. Association constants have not been reported for "bisected" glycopeptides. However, **7** bound to RCA-120–agarose very tightly, as would be predicted from the high association constant reported[32] for "nonbisected" **12**. Similarly, **6** bound weakly to the column, in keeping with a relatively low association constant[32] for **13**.

DISCUSSION

The purpose of this study was to determine the effect of a "bisecting" GlcNAc group on the patterns of elution of complex glycopeptides from Con A–Sepharose, E-PHA–agarose, and RCA-120–agarose columns. This study was prompted by the finding that a "bisecting" GlcNAc group causes dramatic inhibition of the action of several enzymes on glycopeptide substrates[1–6] and by previous indications that the presence of a "bisecting" GlcNAc group causes inhibition of binding to Con A–Sepharose. Table I summarizes the results of our studies; although some of the patterns have been reported previously by this and other laboratories, several interactions are reported herein for the first time.

Con A–agarose. — Previous studies with Con A–Sepharose have shown that tri- and tetra-antennary, complex oligosaccharides are eluted unretarded, that some biantennary, complex oligosaccharides bind weakly to the lectin column and can be eluted with 10–20mM methyl α-D-gluco- or manno-pyranoside, and that strongly adherent oligosaccharides requiring 0.1–0.5M α-D-gluco- or manno-pyranoside for elution are of the high-mannose type[9–14,33,34]. It has also been shown that some biantennary and hybrid oligosaccharides also bind strongly to Con A–Sepharose [6,10,16,35,36]. Interaction with Con A–Sepharose is not affected by the presence or absence of a core α-L-fucopyranosyl group linked (1→6) to the asparagine-linked GlcNAc residue.

The requirements for binding to Con A–Sepharose can therefore be summarized as follows. There must be at least two interacting Man residues attached to a third Man residue. An interacting Man residue is either a terminal, nonreducing group or residue substituted at O-2, thereby explaining the lack of binding of tri- and tetra-antennary, complex oligosaccharides. Binding of "non-bisected", biantennary complex oligosaccharides is relatively insensitive to substitution on the α-D-Man*p*-(1→3) branch, *i.e.*, **22–25** all bound quite tightly (Table I). Interaction is strengthened by a terminal β-D-Glc*p*NAc-(1→2) group on the α-D-Man*p*-(1→6) branch (**4, 14,** and **15**), but is weakened if a Gal or sialyl→Gal group covers the GlcNAc residue on the α-D-Man*p*-(1→6) branch (**3, 12, 13, 17,** and **26**).

Glycopeptides **3, 12, 17,** and **26** bound to Con A–Sepharose in the present study and were eluted with 10mM methyl α-D-glucopyranoside. However, such glycopeptides showed retarded elution with buffer lacking methyl α-D-glucoside in previous studies from this laboratory[10,31]. This result is attributed to variations between different batches of Con A–Sepharose. Also, repeated use of the batch of Con A–Sepharose used in the present work (more than 20 times) weakened the interaction of glycopeptides like **3** with the lectin column, so that they were eluted in a retarded pattern and did not bind to the column. This effect may be due to bleeding of Con A from the column.

A "bisecting" GlcNAc group weakens interaction with Con A–Sepharose (Table I). However, the interaction of "bisected", biantennary complex oligosaccharides was strengthened by the presence of a terminal β-D-Glc*p*NAc-(1→2)

group on the α-D-Man*p*-(1→3) branch (**5, 9**, and **16**) and these glycopeptides were eluted in a retarded pattern (Table I). We found that **5** and **16** were retarded but not bound in previous work[2,4] with other batches of Con A. There has been a report in which these structures bound to Con A–Sepharose, thus indicating batch-to-batch variation[9].

Brisson and Carver[7] have pointed out that the "bisecting" GlcNAc group covers the β-D-Glc*p*NAc-(1→2)-α-D-Man*p*-(1→3) branch, thereby inhibiting the action of four enzymes involved in glycoprotein synthesis[1–6]. It has also been shown that the α-D-Man*p*-(1→6) branch of "bisected", biantennary glycopeptides is folded back towards the core[7]. These steric factors probably explain the inhibition of oligosaccharide binding to Con A–Sepharose. Although both the α-D-Man*p*-(1→3) and -(1→6) branches must be available for optimum binding to Con A–Sepharose, it is clear from the data of Table I that the terminal group of the α-D-Man*p*-(1→6) branch of "nonbisected" structures plays a more decisive role in determining binding than does that of the α-D-Man*p*-(1→3) branch. We do not understand the strengthening of the binding by a terminal β-D-Glc*p*NAc-(1→2) group on the α-D-Man*p*-(1→3) branch of "bisected" glycopeptides structures and on the α-D-Man*p*-(1→6) branch of "nonbisected" glycopeptides. One possible explanation is that a "bisecting" GlcNAc group causes the α-D-Man*p*-(1→6) branch to fold back towards the core[7].

Baenziger and Fiete[9] measured the association constants of various glycopeptides with Con A–Sepharose. There is general agreement between these constants and the data in Table I. For example, a decreased association constant was found upon introduction of a "bisecting" GlcNAc group into **3, 4, 15**, and **23**. However, increased association constants were not detected upon introduction of a terminal GlcNAc group on the α-D-Man*p*-(1→6) branch, as might be expected from the data in Table I.

E-PHA–agarose. — Batch-to-batch variation was also evident for E-PHA–agarose when results from different laboratories are compared[15–18,37]. In our studies, elution from E-PHA–agarose fell into three categories, unretarded (fractions 11,12), slightly retarded (fractions 15–17), and strongly retarded (fractions 38–44). Table I lists the elution profiles of all the structures studied. Both a "bisecting" GlcNAc and a terminal Gal group on the α-D-Man*p*-(1→6) branch were essential for strong interaction with E-PHA–agarose. There is good agreement between our experiments with glycopeptides and those of Yamashita *et al.*[18] with oligosaccharides, showing that the peptide portion does not influence binding to E-PHA. The α-D-Man*p*-(1→3) branch also appeared to play a role in binding to this lectin since removal of the β-D-GlcNAc*p* group linked (1→2) to the α-D-Man*p*-(1→3) branch prevented binding[18].

RCA-120–agarose. — Compounds lacking a terminal Gal group either passed through the RCA-120–agarose column unretarded (**4, 5, 10**, and **13**) or slightly retarded (**6**). It has been reported[19,32] that terminal Gal groups are essential for binding and that RCA I interacts with **2** more strongly than with **15**. We have

verified this finding with RCA-120–agarose and have shown, in addition, that **17** interacts even more weakly than **15**. Glycopeptide **19** did not adhere to our batch of RCA-120–agarose but was eluted in a broad retarded peak at about 68 mL; therefore, it interacted more strongly than **2** (elution at 50 mL). Thus, the interaction with RCA-120–agarose depends directly on the number of terminal Gal groups on the oligosaccharide.

The effect of a "bisecting" GlcNAc group on RCA–agarose binding was investigated by Irimura *et al.*[17] who reported that **8** binds to this lectin column. We have carried these studies further. With our batch of RCA-120–agarose, glycopeptide **8** was retarded rather than bound and, in fact, eluted at a more retarded position (60 mL) than **2** (50 mL), thus showing that the "bisecting" GlcNAc group enhanced binding to a small degree. Except for **7**, which interacted very tightly (elution at 60 mL), both "bisected" and "nonbisected" glycopeptides having a single terminal Gal group were eluted slightly retarded between 13 and 30 mL (Table I). A "bisecting" GlcNAc group caused minor stimulation of binding of **17** and minor inhibition of binding of **15**.

Binding to RCA-120–agarose reflected primarily the number of terminal Gal groups. No binding occured in the absence of a terminal Gal group and binding increased as the number of terminal Gal groups was increased from one to three per mole. The "bisecting" GlcNAc group had only a relatively minor effect on the binding of oligosaccharides to RCA-120–agarose, thus indicating that the steric hindrance of the "bisecting" GlcNAc group affects the β-D-Glc*p*NAc-(1→2)-α-D-Man*p*-(1→3)-β-D-Man*p*-(1→4) portion[1] but does not extend to the terminal Gal group of this branch. This conclusion agrees with three-dimensional studies on "bisected" oligosaccharides[7]. However, binding of "bisected" glycopeptides to RCA-120–agarose was enhanced by the presence of a terminal Gal group on the α-D-Man*p*-(1→6) branch (Table I); this was especially evident for **7**. The interactions of **6** and **7** with RCA-120–agarose supported the finding of Baenziger and Fiete[32] that RCA can interact with a Gal residue substituted at O-6 by a sialyl residue; the relatively poor interactions of **12** and **13**, however, were unexpected.

In conclusion, we have demonstrated several lectin–oligosaccharide interactions not previously reported and have shown that a "bisecting" GlcNAc group affects the binding of glycopeptides to all three lectins studied.

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