THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 256, No. 13, Issue of July 10, pp. 6633-6640,1981
Printed in U.S.A.

The Carbohydrate-binding Specificity of Pea and Lentil Lectins

FUCOSE IS AN IMPORTANT DETERMINANT*

(Received for publication, January 23, 1981, and in revised form, March 16, 1981)

Kerry Kornfeld, Marc L. Reitman, and Rosalind Kornfeld

From the Washington University School of Medicine, Departments of Internal Medicine and Biological Chemistry, St. Louis, Missouri 63110

The carbohydrate-binding specificities of pea lectin and lentil lectin have been determined by testing the ability of radioactively labeled glycopeptides to bind to columns of pea lectin-Sepharose and lentil lectin-Sepharose. The presence of a fucose residue attached to the asparagine-linked *N*-acetylglucosamine residue of the test glycopeptide was essential for high affinity binding to both pea and lentil lectin-Sepharose but not to concanavalin A-Sepharose. In addition to fucose, 2 α-mannosyl residues were required for glycopeptide binding to the pea and lentil lectin-Sepharose columns. Substitution of the α-mannosyl residues at C-2 did not prevent their interaction. Substitution of 1 α-mannosyl residue at both C-2 and C-4 did prevent glycopeptide binding, but substitution of 1 α-mannosyl residue at C-2 and C-6 did not impair binding. Glycopeptide binding to lentil lectin-Sepharose was enhanced by the exposure of terminal *N*-acetylglucosamine residues on the glycopeptide, whereas binding to pea lectin-Sepharose was enhanced by the exposure of terminal mannose residues. The differences in carbohydrate binding specificity of pea lectin-Sepharose and Con A-Sepharose were exploited to fractionate a mixture of [2-^{3}H]mannose-labeled glycopeptides derived from mouse lymphoma cell glycoproteins.

Two mutant mouse lymphoma cell lines, selected for their resistance to pea lectin, were recently found to be defective in fucose metabolism and to have membrane glycoproteins deficient in fucose (1). Both mutant cell lines have a decreased number of high affinity pea lectin-binding sites (2), and in the PL^{R}1.3 cell line, which cannot convert GDP-mannose to GDP-fucose, growth in 10 mM fucose can bypass the block and restore high affinity pea lectin binding (1). These findings strongly implicated fucose as a major determinant of the pea lectin carbohydrate-binding specificity. Previously, on the basis of studies with various mono- and disaccharides, pea lectin had been classified with lentil lectin and concanavalin A as having α-glucosyl- and α-mannosyl-binding-specificity (3, 4). In more recent years, the carbohydrate-binding specificity of both lentil lectin (5-7) and Con A[1] (8, 9) has been examined in more detail using glycopeptides with oligosaccharide chains of varying structure to probe the sugar requirements for tight binding. It was concluded from these studies that both lectins, while requiring α-mannosyl groups, probably interact with multiple sugar residues rather than a single α-mannose residue to promote tight binding. However, in none of these studies had fucose been recognized as a determinant, although many of the test glycopeptides did contain fucose residues in the core region of their oligosaccharide chains. Accordingly, we undertook to reexamine the high affinity carbohydrate-binding specificity of pea and lentil lectins by testing the ability of various radioactively labeled glycopeptides to bind to columns of pea lectin-Sepharose and lentil lectin-Sepharose. We found that fucose residues are indeed an important determinant in tight binding to pea and lentil lectin but not to Con A.

* This investigation was supported in part by Grant R01 CA 08759 from the United States Public Health Service and by National Institutes of Health Research Service Award GM 07200, Medical Scientist, from the National Institute of General Medical Sciences. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] The abbreviations used are: Con A, concanavalin A; Fuc, fucose; RCAI, *Ricinus communis* agglutinin I (from castor beans); TBS, 0.15 M NaCl, 0.01 M Tris, pH 8.0, 1 mM $CaCl_2$, 1 mM $MgCl_2$. All sugars are of the D configuration, except fucose, which is of the L form.

EXPERIMENTAL PROCEDURES

Materials—Sephadex G-25-80, Con A-Sepharose, and lentil lectin-Sepharose were obtained from Pharmacia Fine Chemicals. RCAI-Sepharose was prepared as described by Adair and Kornfeld (10). Pea lectin-Sepharose, obtained from Dr. I. Trowbridge of the Salk Institute, was prepared by him as described previously (11). D-[2-^{3}H] Mannose (18.4 Ci/mmol) and [^{3}H]acetic anhydride (100 mCi/mmol) were from New England Nuclear. *Vibrio cholera* neuraminidase was obtained from Calbiochem-Behring Corp. β-Galactosidase, β-*N*-acetylglucosaminidase, and α-mannosidase was prepared from jack bean meal by the method of Li and Li (12). Purified diplococcal β-galactosidase was a gift from Dr. Jacques Baenziger (Washington University), and bovine epididymal α-fucosidase was purchased from Sigma. Amberlite MB-3 was obtained from Chemical Dynamics Corp., South Plainfield, NJ, and silica gel G (250 microns)-coated thin layer chromatography plates were from Analtech, Inc. The 3a70 scintillation mixture was obtained from Research Products International Corp., Elk Grove, IL.

Preparation of Labeled Human IgG Glycopeptides—The glycopeptide fraction from human myeloma IgG (M^{c}M) was isolated and characterized as described previously (13, 14). Its average structure is shown in Fig. 1. The glycopeptide fraction was radioactively labeled by acetylation of the peptide amino groups with [^{3}H]acetic anhydride in 0.5% $NaHCO_3$ and then passed over a column of Sephadex G-25 to separate it from reactants. When this ^{3}H-acetylated glycopeptide fraction was passed over RCAI-Sepharose, it separated into 3 major and 1 minor components as shown in Fig. 2. The structures of the glycopeptides in peaks 1, 3, and 4 were deduced from sequential glycosidase digestion and are also shown in Fig. 1. It can be seen that the structure determined for the unfractionated IgG glycopeptide represents the average of its component parts, which display microheterogeneity in their outer chain galactose content, accounting for their separation by RCAI, a β-galactosyl-binding lectin (15). The minor peak 2 glycopeptide is the sialylated form of peak 4. A series of glycopeptides with shorter oligosaccharide chains were derived from the peak 3 or G-2 glycopeptide by sequential or combined treatment with various exoglycosidases as described in detail previously (8). Table I shows the enzyme degradations and the structure of the products obtained.

Sources of Other Test Glycopeptides—Fig. 3 shows the structures of two other immunoglobulin glycopeptides that bound to pea and lentil lectin-Sepharose columns. The glycopeptide from rat IgE was

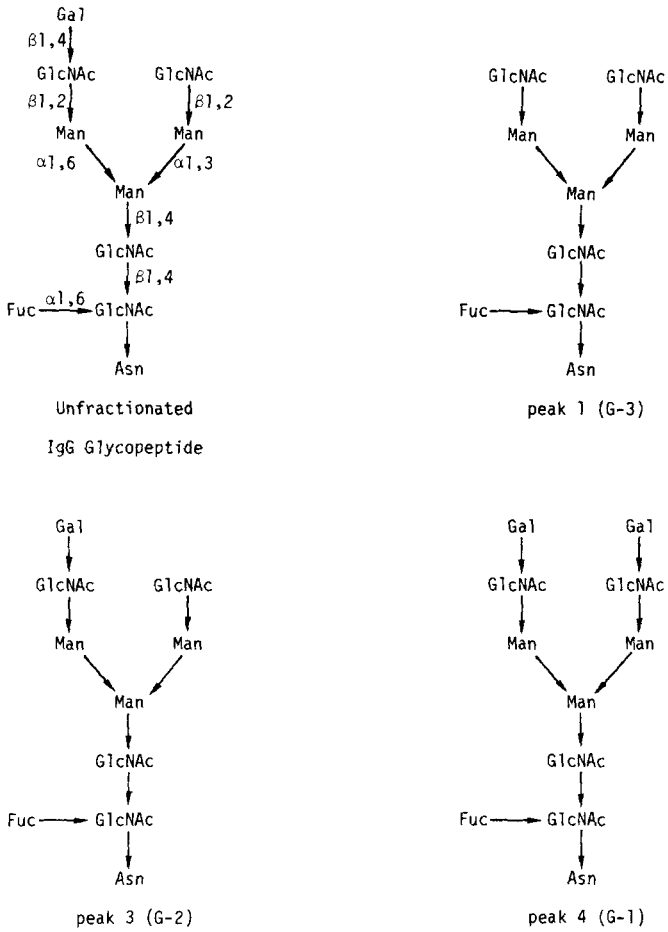


FIG. 1. **Structures of the unfractionated IgG glycopeptide and its constituent glycopeptides obtained by fractionation on RCAI-Sepharose (Fig. 2).**

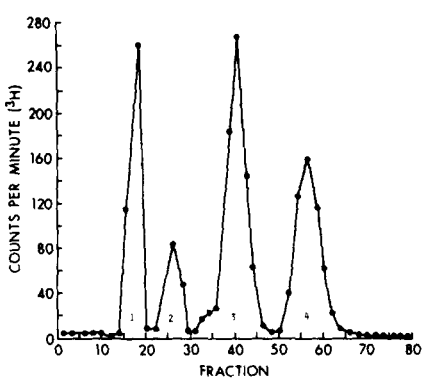
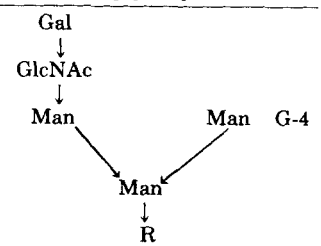
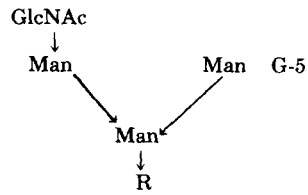
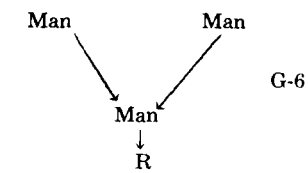
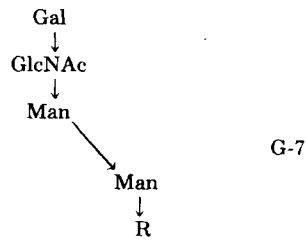


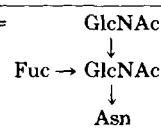
FIG. 2. **Fractionation of IgG glycopeptides on RCAI-Sepharose.** The ^{3}H-acetylated IgG glycopeptide was loaded on a column (1 × 50 cm) of RCAI-Sepharose and eluted with phosphate-buffered saline, pH 7.4. Two-ml fractions were collected and radioactivity was measured in 20-μl aliquots of every other fraction. Peaks *1*, *2*, *3*, and *4* were pooled as shown and then desalted on Sephadex G-25.

provided by Dr. Jim Rearick of Washington University and the mouse IgM glycopeptide 1B was isolated and characterized as described previously (16). Both were metabolically labeled with [2-^{3}H]mannose. Fig. 4 shows the structure of six glycopeptides that did not bind to pea and lentil lectin-Sepharose columns. The mouse IgM glycopeptide 1A was isolated and characterized as described previously (16). The high mannose glycopeptides from Chinese hamster ovary cells containing 5 and 8 mannose residues (17) and the high mannose glycopeptide from Thy-1$^-$e mouse lymphoma cells (18) were supplied by Dr. Stuart Kornfeld of Washington University, St. Louis, MO. These four glycopeptides were metabolically labeled with [2-^{3}H]mannose.

TABLE I

Structure of partially degraded test glycopeptides derived from IgG glycopeptide G-2

Enzyme treatment	Glycopeptide product[a]
β-*N*-Acetylglucosaminidase	 G-4
β-*N*-Acetylglucosaminidase and then β-galactosidase (Diplococcal)	 G-5
β-*N*-Acetylglucosaminidase + β-galactosidase (Diplococcal)	 G-6
β-*N*-Acetylglucosaminidase + α-mannosidase	 G-7

[a] R = 

The two fucosylated asialotransferrin glycopeptides were prepared by pronase digestion of ^{14}C-fucosylated asialotransferrin with fucose either in α1 → 2 linkage to the terminal galactose or in α1 → 3 linkage to the subterminal *N*-acetylglucosamine residues. These molecules were synthesized by Beyer *et al.* (19) and kindly provided to us by Dr. Tom Beyer of Duke University, Durham, NC.

Affinity Chromatography on Pea Lectin-Sepharose and Lentil Lectin-Sepharose—The carbohydrate-binding specificity of pea and lentil lectins were assayed by testing the ability of each lectin-Sepharose conjugate to bind the various radioactive test glycopeptides. A qualitative estimate of relative affinity was provided by the concentration of haptene sugar required to elute the glycopeptide. The standard test system consisted of a column (0.7 × 4 cm) of either pea lectin-Sepharose or lentil lectin-Sepharose onto which the radioactive glycopeptide was loaded in Tris-buffered saline containing 0.15 M NaCl, 0.01 M Tris, pH 8.0, 1 mM $CaCl_2$, and 1 mM $MgCl_2$. The elution program was 10 ml of TBS, then 20 ml of TBS containing 10 mM α-methyl glucoside, and finally 20 ml of TBS containing 0.5 M α-methyl mannoside. One-ml fractions were collected directly into 6-ml counting vials, to which 3 ml of 3a70 scintillation mixture were added before counting. The columns were run at room temperature. Different amounts of radioactivity were applied to the column in different experiments. The recovery of radioactivity from the columns in all cases ranged from 97 to 100% of that loaded.

Preparation of [2-^{3}H]Mannose-labeled Whole Cell Glycopeptides—The BW5147 mouse lymphoma cell line was cultured, labeled with [2-^{3}H]mannose for 2 days, and extracted as described previously (1). The pellet was digested with pronase and the resulting glycopeptides were desalted by gel filtration on Sephadex G-25 (1).

Methods of Glycopeptide Structural Analysis—The [2-^{3}H]mannose and fucose content of labeled glycopeptides was determined after acid hydrolysis in 2 N H_2SO_4 for 4 h at 100 °C as described previously (1). The [2-^{3}H]mannose-labeled glycopeptides were methylated by the method of Hakomori (20), and the methylated mannose and fucose species obtained after hydrolysis were separated by thin layer chromatography as described previously (21). Periodate oxidation of [2-^{3}H]mannose-labeled glycopeptides was carried out as follows. Duplicate samples of each glycopeptide were treated with 0.08 M sodium metaperiodate in 0.05 M acetate buffer, pH 4.6, for 20 h at 4 °C in the dark in a final volume of 40 μl. Ethylene glycol (15 μl of 0.8 M) was added to consume the excess periodate, and the samples were incubated at room temperature for 2 h. Next, nonradioactive carrier mannose (0.2 μmol) was added to both the reaction mixtures and to duplicate samples of each glycopeptide which had not been subjected to oxidation and which served as controls. All samples were hydrolyzed in 2 N H_2SO_4 for 4 h at 100 °C, diluted with 4 volumes of water, and deionized by passage over a small column (0.5 × 6 cm) of Amberlite MB-3 mixed-bed resin which was washed with approximately 10 ml of water to elute neutral sugars. The samples were concentrated and subjected to descending chromatography on Whatman No. 1 paper in pyridine/ethyl acetate/H_2O (1:3.6:1.15, upper phase) for 18 h. The migration positions of standard mannose and fucose on separate lanes were revealed by the $AgNO_3$ dip method (22), and the corresponding mannose areas from each sample lane were eluted with water. One aliquot of each eluate was counted for radioactivity and another was analyzed for mannose content as described previously (13). The amount of [2-^{3}H]mannose in each sample was calculated based on the recovery of the cold carrier mannose. Glycosidase digestions of glycopeptides were carried out under the following conditions: *V. cholera* neuraminidase, 250 units/ml in 0.05 M acetate buffer, pH 4.6, 0.15 M NaCl, 10 mM $CaCl_2$ at 37 °C for 72 h; jack bean β-galactosidase, 0.4 unit/ml, β-*N*-acetylglucosaminidase, 1.3 units/ml, and α-mannosidase, 16 units/ml in 0.05 M sodium citrate buffer, pH 4.6, at 37 °C for 48 h; *Diplococcal* β-galactosidase, 120 milliunits/ml in 0.1 M cacodylate buffer, pH 6.0, at 37 °C for 24 h; and bovine epididymal α-fucosidase, 0.3 units/ml in 0.025 M sodium citrate, pH 4.5, at 37 °C for 48 h. The digestions were stopped by putting the reaction tubes into boiling water for 2 min.

RESULTS

Fucose Is Required for Glycopeptide-binding to Pea and Lentil Lectins—Fig. 5 shows the elution profiles on pea and lentil lectin-Sepharose obtained for the unfractionated IgG glycopeptide. In both cases, the sample bound to the lectin-Sepharose, and could be eluted with either 10 mM α-methyl glucoside in the case of the pea lectin-Sepharose (Fig. 5*A*) or 0.5 M α-methyl mannoside in the case of the lentil lectin-Sepharose (Fig. 5*C*). In contrast, after the glycopeptide was treated with α-fucosidase, it no longer bound to either the pea or lentil lectin-Sepharose column and emerged from both in the TBS wash fractions (Figs. 5*B* and *D*).

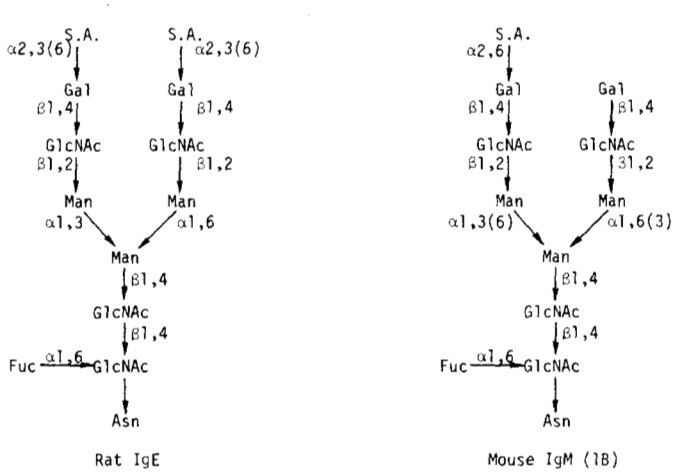


FIG. 3. **Structures of two glycopeptides, metabolically labeled with [2-^{3}H]mannose, that bind to pea and lentil lectin-Sepharose columns.** See "Experimental Procedures" for sources. *S.A.*, sialic acid.

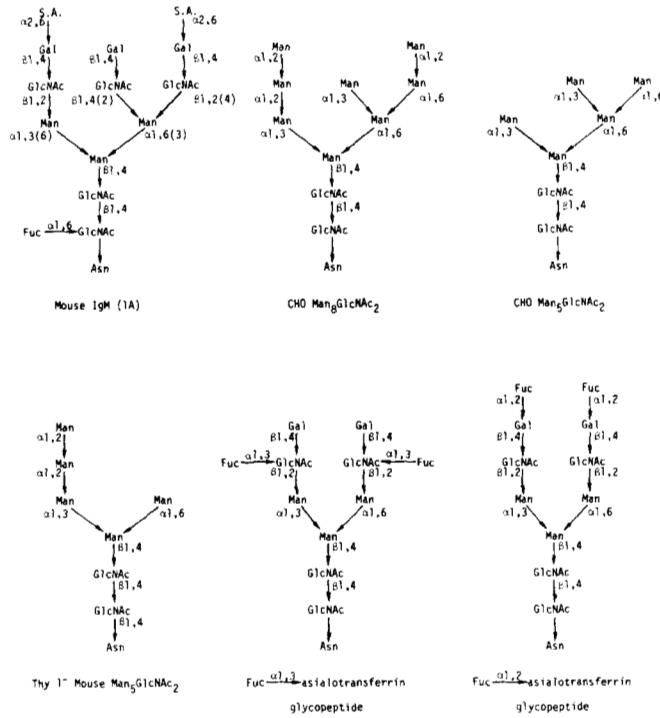


FIG. 4. **Structures of six glycopeptides that cannot bind to pea or lentil lectin-Sepharose columns.** See under "Experimental Procedures" for sources of each. *CHO*, Chinese hamster ovary. *S.A.*, sialic acid.

Panel *E* of Fig. 5 shows that the untreated IgG glycopeptide also binds to Con A-Sepharose. When the defucosylated glycopeptides in the TBS wash fractions from both the pea and lentil lectin-Sepharose columns were tested for their ability to bind to a column of Con A-Sepharose, they were able to bind and were eluted with 10 mM α-methyl glucoside as shown in panel *F*. This indicated that the failure of the enzyme-treated glycopeptide to bind to pea or lentil lectin was due to removal of fucose residues and not due to other degradation of the oligosaccharide, such as cleavage by an endo-β-*N*-acetylglucosaminidase activity. These findings demonstrated that both pea and lentil lectin-Sepharose require fucose in the glycopeptide for high affinity binding, whereas Con A-Sepharose does not.

Effect of Outer Chain Sugar Residues on Glycopeptide Binding to Pea and Lentil Lectin-Sepharose—To further examine the contribution of various sugar residues to the binding of glycopeptides to pea and lentil lectin, the fractionated IgG glycopeptides shown in Fig. 1 and the series of modified IgG glycopeptides shown in Table I were tested. Fig. 6 shows the elution profiles obtained on pea and lentil lectin-Sepharose columns with these glycopeptides. Panels *A* and *E* show that glycopeptide G-1, with two terminal galactose residues, binds to both lectins. It is eluted promptly from the pea lectin column with 10 mM α-methyl glucoside (Fig. 6*A*), but is eluted more slowly from the lentil lectin column, so that some glycopeptide is still on the column when the 0.5 M α-methyl mannoside elution is begun, producing a spike on the trailing edge of the elution profile (Fig. 6*E*). When the sialylated rat IgE and mouse IgM 1B glycopeptides (Fig. 3) were tested, they gave elution profiles identical with those shown for G-1 in panels *A* and *E*. This indicates that substitution of 1 or both of the terminal galactose residues with sialic acid does not alter the affinity of the glycopeptide for pea or lentil lectin. Panels *B* and *F* show that glycopeptide G-2, with 1 terminal

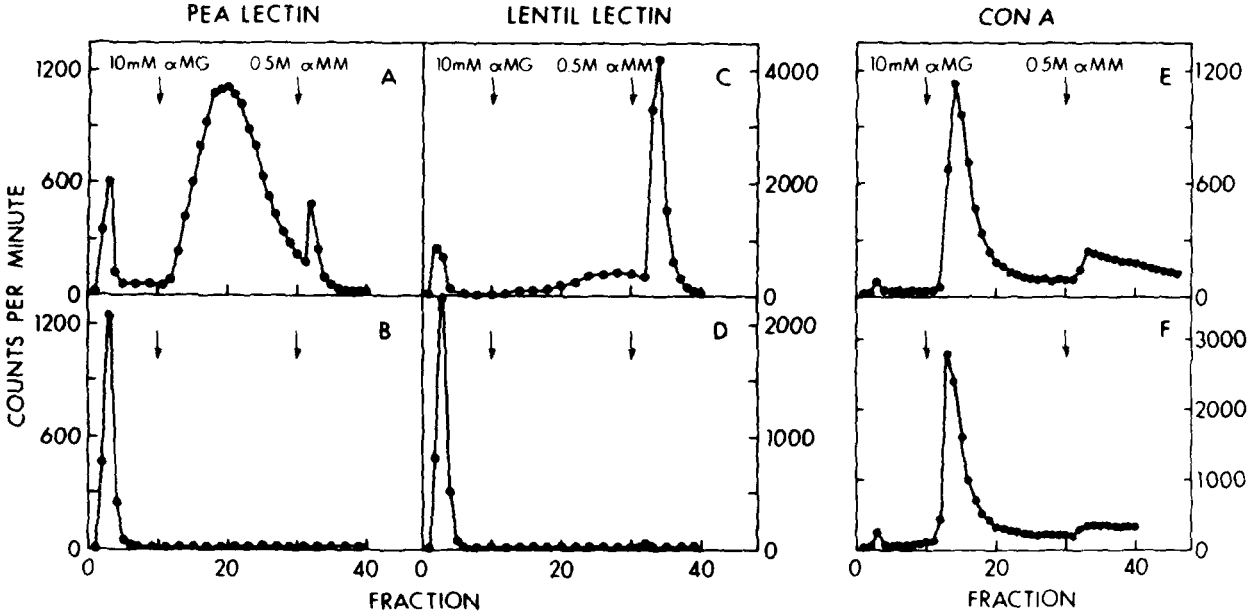


FIG. 5. **Elution profiles of unfractionated IgG glycopeptides on lectin-Sepharose columns before and after treatment with α-fucosidase.** In panels *A*, *C*, and *E* are the elution profiles of the IgG glycopeptide on pea lectin, lentil lectin, and Con A-Sepharose columns, respectively. In panels *B* and *D* are the elution profiles of α-fucosidase-treated glycopeptide on pea and lentil lectin-Sepharose, respectively. When the α-fucosidase treatment was repeated with larger amounts of the IgG glycopeptide and only a portion of each fraction from the pea and lentil lectin-Sepharose columns was counted, the same profiles as *B* and *D* were obtained. The defucosylated glycopeptide in the TBS wash fractions was then put over Con A-Sepharose columns. In panel *F* is the elution profile of the TBS wash from the lentil lectin-Sepharose. The TBS wash from the pea lectin-Sepharose gave the same profile. The *arrows* show where elution was begun with α-methyl glucoside (*αMG*) and α-methyl mannoside (*αMM*). The small amount of radioactive glycopeptide (approximately 10% of the total) which passed through the columns in panels *A* and *C* bound to Con A-Sepharose. This material is presumed to be nonfucosylated glycopeptide present in the untreated sample.

galactose and 1 terminal *N*-acetylglucosamine residue, also binds to both lectins and is eluted promptly from the pea lectin column with 10 mM α-methyl glucoside (Fig. 6*B*) but is not eluted from the lentil lectin column until the 0.5 M α-methyl mannoside is added (Fig. 6*F*). Glycopeptide G-3 gave elution profiles on both columns identical with those of G-2, suggesting that the presence of either 1 or 2 terminal *N*-acetylglucosamine residues enhances glycopeptide binding to lentil lectin-Sepharose but not to pea. Panels *C* and *G* show the elution profiles of glycopeptide G-4, which has 1 terminal mannose residue and 1 terminal galactose residue, having been derived from glycopeptide G-2 by removal of a terminal *N*-acetylglucosamine to expose the underlying mannose residue (Table I). This structural alteration results in enhanced binding of glycopeptide G-4 to pea lectin-Sepharose, as evidenced by its delayed elution with 10 mM α-methyl glucoside (Fig. 6*C*), and diminished binding of glycopeptide G-4 to lentil lectin-Sepharose, as shown by its prompt elution with 10 mM α-methyl glucoside (Fig. 6*G*). Glycopeptides G-5 and G-6 (see Table I) gave elution profiles on both columns essentially identical with those of G-4, suggesting that the presence of either 1 or 2 terminal mannose residues enhances glycopeptide binding to pea lectin, whereas removal of terminal *N*-acetylglucosamine residues diminished binding to lentil lectin. Panels *D* and *H* in Fig. 6 show the elution profile obtained with glycopeptide G-7, which was derived from G-4 by removal of the terminal mannose residue. This glycopeptide was unable to bind to either column, indicating that both pea and lentil lectin-Sepharose, like Con A-Sepharose (23), require a glycopeptide structure with 2 interacting α-mannosyl residues for tight binding.

Glycopeptides That Cannot Bind to Pea or Lentil Lectin-Sepharose Columns—None of the glycopeptides shown in Fig. 4 bound to pea and lentil lectin-Sepharose columns. The mouse IgM glycopeptide 1A, which differs from the IgG glycopeptides in having a third outer chain arising from an α-mannose residue substituted at C-2 and C-4, is also unable to bind to Con A-Sepharose (16). Thus pea, lentil, and Con A-Sepharose are alike in being unable to interact with an α-mannosyl residue substituted at both C-2 and C-4. Although α-mannose residues are important for glycopeptide binding to both pea and lentil lectin-Sepharose, the presence of many α-mannose residues will not overcome the absence of a fucose residue in the core, as shown by the fact that none of the three high mannose glycopeptides shown in Fig. 4 was able to bind to either lectin-Sepharose column. The fact that the asialotransferrin glycopeptides with fucose linked either α1,3 to the outer chain *N*-acetylglucosamine residues or α1,2 to the outer chain galactose residues could not bind to pea and lentil lectin-Sepharose shows that α-fucose residues in those positions, unlike fucose linked α1,6 to the core *N*-acetylglucosamine residue, do not promote tight binding.

Use of Lectin-Sepharose Columns to Fractionate a Mixture of Glycopeptides—The different binding specificities found for pea and lentil lectin-Sepharose as compared to Con A-Sepharose suggested that sequential use of these lectin-Sepharose columns could provide superior fractionation of a mixture of glycopeptides. Accordingly, [2-^{3}H]mannose-labeled glycopeptides derived from mouse lymphoma cells as described under "*Experimental Procedures*" were sequentially fractionated on columns of Con A-Sepharose and pea lectin-Sepharose. Fig. 7 shows that Con A-Sepharose resolved this mixture into several fractions: fraction 1 which did not bind, fraction 2 which eluted with 10 mM α-methyl glucoside, and a third fraction that eluted with 0.5 M α-methyl mannoside. The latter fraction was shown to consist of high mannose type glycopeptides, 90% of which were susceptible to cleavage with endo-β-*N*-acetylglucosaminidase C$_{II}$, liberating a series of oli-

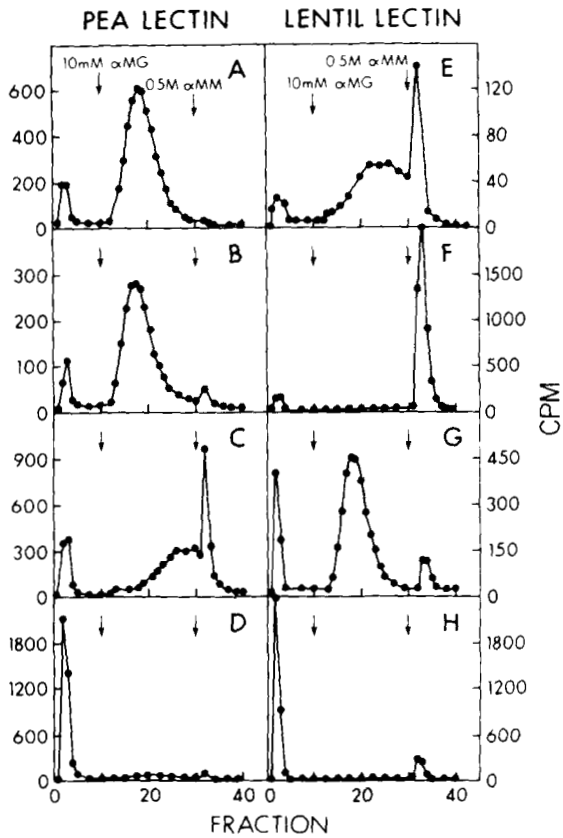


FIG. 6. **Elution profiles of glycopeptides on pea and lentil lectin-Sepharose.** Glycopeptide G-1 (*A* and *E*); glycopeptide G-2 (*B* and *F*); glycopeptide G-4 (*C* and *G*); and glycopeptide G-7 (*D* and *H*) were passed over pea lectin-Sepharose (*A–D*) and lentil lectin-Sepharose (*E–H*) as described under "Experimental Procedures." The *arrows* show where elution was begun with α-methyl glucoside (*αMG*) and α-methyl mannoside (*αMM*).

gosaccharides containing 5 to 9 mannose residues. Fraction 1, presumed to consist of complex type glycopeptides with more than two outer chains (*i.e.* disubstituted α-mannose residues), was passed through a pea lectin-Sepharose column and resolved into two fractions, 1A and 1B, as shown in the lower panel of Fig. 7. Fraction 1A did not bind and contained about 60% of the ^{3}H label, which after acid hydrolysis, was shown to consist of both [^{3}H]fucose and [^{3}H]mannose in a ratio of 1.0/3.0. Fraction 1B eluted with 10 mM α-methyl glucoside and contained about 40% of the ^{3}H label, which was also shown to consist of [^{3}H]fucose and [^{3}H]mannose in a ratio of 1.0/3.0. These results indicated that differences in fucose content could not explain the different pea lectin-binding affinities of glycopeptides 1A and 1B, and further studies were performed, as described below, to define the structural differences of 1A and 1B. When fraction 2 from the Con A-Sepharose column, presumed to consist of complex type glycopeptides with two outer chains, was passed over pea lectin-Sepharose (data not shown), 27% of the ^{3}H label did not bind (fraction 2A), and 73% of the ^{3}H label bound and was eluted with 10 mM α-methyl glucoside (fraction 2B). Fucose (up to 1 M) did not elute fraction 2B from pea lectin-Sepharose (data not shown). When 2A and 2B were hydrolyzed, it was found that 2B contained [^{3}H]fucose and [^{3}H]mannose in a ratio of 1.1/3.0, whereas 2A had less than 0.2 [^{3}H]fucose per 3.0 [^{3}H]mannose residues, thus accounting for its inability to bind to pea lectin-Sepharose.

Structural Studies on Glycopeptides 1A and 1B—The fact that the multibranched glycopeptides in fraction 1 from Con A-Sepharose could be further fractionated on pea lectin-Sepharose suggested that heterogeneity existed in the branching patterns of the α-mannose residues. Accordingly, structural studies were performed to elucidate the branching patterns of 1A and 1B. When 1A and 1B were methylated, and then hydrolyzed and the methylated [^{3}H]mannose and [^{3}H]fucose species separated by thin layer chromatography, the results shown in Fig. 8 were obtained. Both glycopeptide 1A, in the top panel, and 1B in the bottom panel, gave rise to species that co-migrated with authentic 3,6-dimethyl-, 2,4-dimethyl-, and 3,4,6-trimethyl mannose and 2,3,4-trimethyl fucose in the ratio of 1.7/1.0/0.3/1.0 in the case of 1A, and in the ratio of 1.0/0.9/1.0/1.1 in the case of 1B. These results are compatible with the earlier finding that in both 1A and 1B, one-fourth of the radioactivity occurs as [^{3}H]fucose, and three-fourths occurs as [^{3}H]mannose. The usual complex type oligosaccharides contain a core β-mannose residue substituted at C-3 and C-6 by α-mannose residues, and this β-mannose residue gives rise to 2,4-dimethyl mannose after methylation. Both 1A and 1B were digested with neuraminidase, β-galactosidase, and β-*N*-acetylglucosaminidase to strip off the outer chains, and the core glycopeptides were isolated and methylated. In both cases, one-fourth of the radioactivity migrated with 2,4-dimethyl mannose on thin layer chromatography and the other three-quarters migrated with 2,3,4,6-tetramethyl mannose and 2,3,4-trimethyl fucose, which have the same mobility in this thin layer system. In another set of experiments, shown in

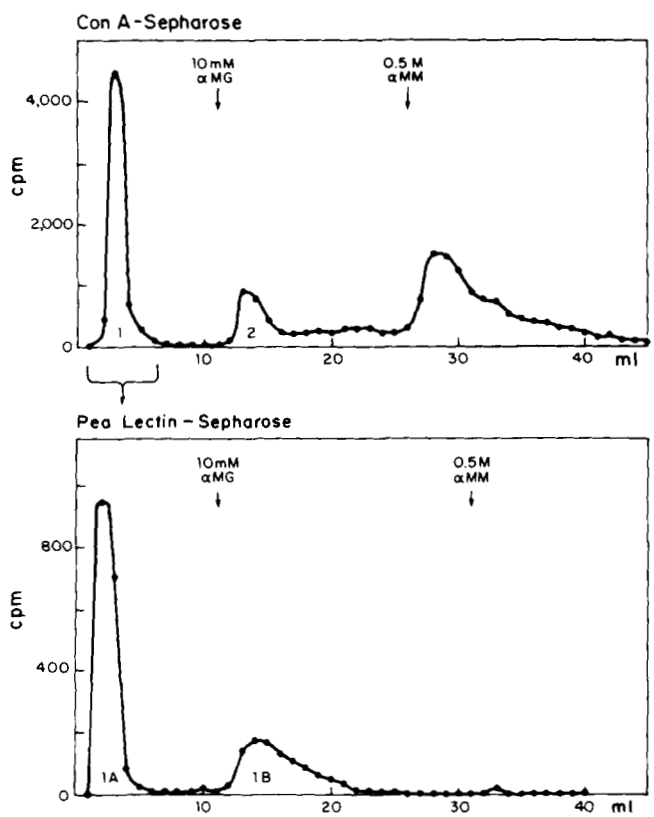


FIG. 7. **Fractionation of [2-^{3}H]mannose-labeled mouse lymphoma cell glycopeptides.** The [2-^{3}H]mannose-labeled glycopeptides were loaded on a column (0.7 × 4 cm) of Con A-Sepharose and eluted as described under "Experimental Procedures" for the pea and lentil lectin-Sepharose columns, and 1-ml fractions were collected. Fractions 2 to 6 were pooled for peak *1*, and fractions 12 to 16 were pooled for peak *2*. The material in peak *1* was then passed over a column (0.7 × 4 cm) of pea lectin-Sepharose and eluted as shown. The *arrows* show where elution was begun with α-methyl glucoside (*αMG*) and α-methyl mannoside (*αMM*).

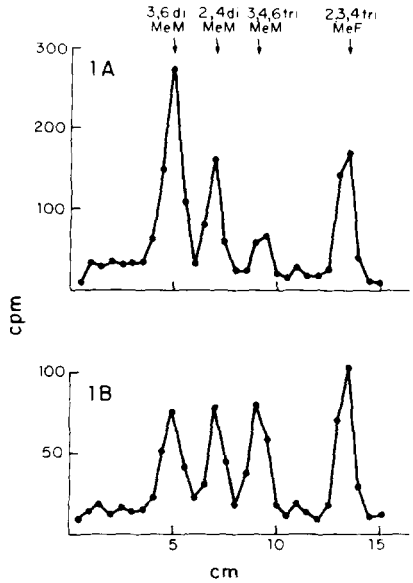


FIG. 8. **Thin layer chromatography of the methylated [2-^{3}H]mannose and fucose species from methylated glycopeptides 1A and 1B.** The hydrolysates of methylated 1A and 1B were spotted on silica gel G thin layer plates and developed in benzene/acetone/H_2O/NH_4OH (50:200:3:1.5) as described under "Experimental Procedures." The *arrows* show the migration positions of authentic methylated mannose (*MeM*) and fucose (*MeF*) standards.

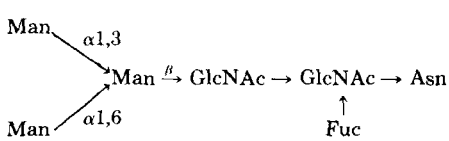
TABLE II

Enzyme digestions of glycopeptides 1A and 1B from mouse lymphoma cells

Enzyme treatment	Radioactive mannose released (residue[a])	
	1A	1B
	%	
1. α-Mannosidase	6.4 (0.3)	2.6 (0.1)
2. β-*N*-Acetylglucosaminidase + α-mannosidase	14.5 (0.6)	8.4 (0.3)
3. β-Galactosidase + β-*N*-acetylglucosaminidase + α-mannosidase	22.4 (0.9)	10.6 (0.4)
4. Neuraminidase + β-galactosidase + β-*N*-acetylglucosaminidase + α-mannosidase	50 (2.0)	50 (2.0)

[a] One residue of mannose is set equal to 25% of the total counts per min, since in both 1A and 1B, 75% of the total counts per min were identified as [^{3}H]mannose and 25% were identified as [^{3}H]fucose.

Table II, 1A and 1B were digested with α-mannosidase alone or in the presence of other glycosidases, and the release of free mannose was measured by paper chromatography of the digests. The results in experiment 4 indicate that digestion with α-mannosidase in the presence of neuraminidase, β-galactosidase, and β-*N*-acetylglucosaminidase liberates 2.0 residues of α-linked mannose from both 1A and 1B. Thus it appears that both 1A and 1B have the usual core structure



and differ from one another in number and linkage of their outer chains. The methylation results indicate that in 1B 1 α-mannose residue is substituted at C-2, giving rise to 3,4,6-trimethyl mannose, and the other α-mannose residue is substituted at C-2 and C-4, giving rise to 3,6-dimethyl mannose. However, this substitution pattern is exactly that found in the mouse IgM glycopeptide 1A, which did not bind to pea lectin-Sepharose. The suspicion then arose that in this thin layer chromatography system, some other dimethyl mannose has the same mobility as 3,6-dimethyl mannose. The only other dimethyl mannose standards tested, namely 2,3- and 2,4-dimethyl mannose, migrate faster than 3,6-dimethyl mannose at 5.8 and 7 cm, respectively. Standards of all four trimethyl mannose species have been tested and found to migrate in discrete positions between 9 and 12 cm on the thin layer chromatogram shown in Fig. 8. However, the relative mobilities of 2,6-, 3,4-, and 4,6-dimethyl mannose in this system are not known, and any or all of them could co-migrate with 3,6-dimethyl mannose. To resolve this identification problem, glycopeptides 1A and 1B were subjected to periodate oxidation, which will destroy any mannose residue which has two unsubstituted vicinal hydroxyl groups. As shown in Table III, periodate oxidation of glycopeptide 1B destroyed 2 of the 3 mannose residues. Since the core β-mannose, substituted at C-3 and C-6, is resistant to oxidation, both α-mannose residues must be susceptible to oxidation and therefore neither of them could be substituted at C-2 and C-4. The α-mannose residue substituted at C-2 which gave rise to the 3,4,6-trimethyl mannose is expected to be oxidized, having vicinal hydroxyl groups at C-3 and C-4. The other α-mannose residue must be substituted at C-2 and C-6 and must have produced 3,4-dimethyl mannose after methylation, because substitution at C-3 and C-4 (giving 2,6-dimethyl mannose) or at C-2 and C-3 (giving 4,6-dimethyl mannose) would protect it from oxidation.

In contrast to glycopeptide 1B, glycopeptide 1A appears to contain a mixture of structures, 30% having three and 70% having four outer branches, since only 0.3 residue of 3,4,6-trimethyl mannose and 1.7 residues of dimethyl mannose migrating with 3,6-dimethyl mannose were found (Fig. 8). Periodate oxidation of glycopeptide 1A (Table III) destroyed 0.8 mannose residue, which can be accounted for by the 0.3 residue of C-2-substituted mannose plus another 0.5 residue of mannose substituted at C-2 and C-6. Thus, about 1.2 residues of mannose must be substituted either at C-2 and C-4, giving rise to 3,6-dimethyl mannose, or possibly at C-2/C-3 and/or C-3/C-4, if in fact 4,6- or 2,6-dimethyl mannose also migrate like 3,6-dimethyl mannose. Fig. 9 shows the structures postulated for the inner regions of glycopeptides 1A and 1B, making the assumption in the case of 1A that the material migrating with 3,6-dimethyl mannose contains only 3,6-dimethyl and 3,4-dimethyl mannose.

Both 1A and 1B contain outer chain sialic acid and galactose residues covering the *N*-acetylglucosamine residues. As shown in Table II, treatment of intact 1A and 1B with α-mannosidase alone released very little free mannose, and treatment with both α-mannosidase and β-*N*-acetylglucosaminidase released less than 1 residue of mannose, indicating that few outer chains terminate in *N*-acetylglucosamine. In contrast, treatment with α-mannosidase in the presence of both β-galactosidase and β-*N*-acetylglucosaminidase released 0.9 residue of mannose from 1A and 0.4 residue from 1B, indicating that a substantial proportion of outer chains terminated in galactose. However, as shown in experiment 4, treatment with neuraminidase was required to get complete degradation of the

TABLE III

Periodate oxidation of glycopeptides 1A and 1B

Sample	Mannose recovered		
	cpm	*% control*	*Residues*
1A Control	7230		3.0
Oxidized	5335	73.8	2.2
1B Control	7640		3.0
Oxidized	2830	37.1	1.1

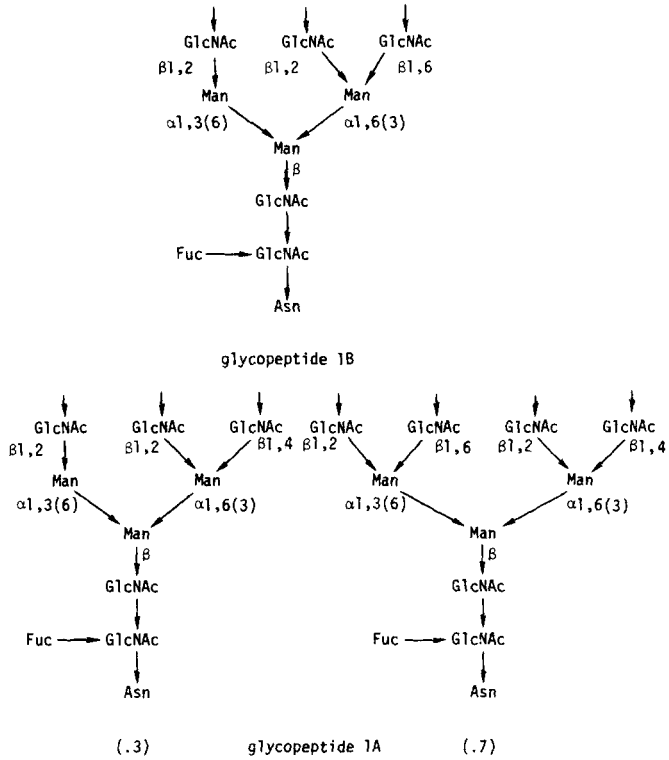


FIG. 9. **Structures proposed for glycopeptides 1A and 1B.**

outer chains and release of 2 full residues of mannose from both 1A and 1B. When glycopeptides 1A and 1B were tested for their ability to bind to lentil lectin-Sepharose, 1A did not bind and 1B did bind and was eluted with 10 mM α-methyl glucoside, indicating that lentil lectin can also interact with an α-mannosyl residue substituted at C-2 and C-6.

DISCUSSION

The observation that mutant mouse lymphoma cell lines resistant to pea lectin have deficient fucosylation of their glycoproteins (1) first implicated fucose as an important determinant in the carbohydrate-binding specificity of pea lectin. The present study confirms that fucose residues in the core region of the immunoglobulin test glycopeptides are the *sine qua non* for binding to both pea and lentil lectin-Sepharose. Although fucose is essential for binding, it is not the sole determinant, and free fucose cannot elute bound glycopeptides from these columns. The α-mannose residues of the test glycopeptides are also required for binding, and 2 α-mannose residues capable of interacting must be available. Like Con A-Sepharose, the pea and lentil lectin-Sepharose could interact with glycopeptides in which both α-mannose residues were substituted at C-2 but not with those in which 1 α-mannose was substituted at C-2 and C-4. In contrast to Con A, both pea and lentil lectins could interact with the glycopeptide 1B from mouse lymphoma cells in which 1 α-mannose residue was substituted at C-2 and C-6. In every case, pea and lentil lectin-Sepharose were able to bind (or not bind) the same glycopeptides, demonstrating the remarkable similarity in their carbohydrate-binding specificity. However, the elution profiles indicated that their relative affinities for some glycopeptides were different. Thus the presence of terminal *N*-acetylglucosamine residues in glycopeptides enhanced their affinity for lentil lectin-Sepharose but not for pea, whereas the presence of terminal mannose residues in glycopeptides enhanced their affinity for pea lectin-Sepharose but not for lentil.

The results from this study are compatible with the findings in earlier studies of lentil lectin interaction with various glycopeptides, and also serve to explain the basis for previously inexplicable effects. Kornfeld *et al.* (5) found that an IgG glycopeptide was the most effective haptene inhibitor of lentil lectin-induced erythrocyte agglutination and that transferrin glycopeptide, which has no fucose, and a $Man_5GlcNAc_2$ glycopeptide were 15-fold poorer as inhibitors. They also observed that removal of the outer chain galactose and *N*-acetylglucosamine residues of the IgG diminished its inhibitory activity. Similar findings were reported by Toyoshima *et al.* (7). Young and Leon (6) tested various glycopeptides for their ability to inhibit lentil lectin precipitation of α-2-macroglobulin, and found that ovalbumin glycopeptides (high mannose type oligosaccharides) did not inhibit, transferrin glycopeptide was a poor inhibitor, and the mixed glycopeptides from a human IgM inhibited strongly. Since only the latter glycopeptides contain fucose residues in the core region of their complex type oligosaccharide chains (24), those findings are in accord with the demonstration in this study of the importance of fucose for high affinity binding. Allen *et al.* (3) found that ovalbumin glycopeptide was a strong inhibitor of Con A, but a much poorer inhibitor of pea, lentil, and fava bean lectins, and that transferrin glycopeptide inhibited all four lectins. Since they tested no fucose-containing glycopeptide, it is not possible to estimate the relative potency of transferrin glycopeptide. In the same study, a variety of monosaccharide derivatives and disaccharides were also tested, and the patterns of inhibition seen for the pea, lentil, and fava bean lectins were very similar to one another but differed from that of Con A.

The similarity of the sugar-binding specificity of pea, lentil, and fava bean lectins is not unexpected, since recent studies have shown that these lectins are also closely related in primary structure and subunit assembly. All three lectins have a structure consisting of two identical subunits which each contain an α- and β-polypeptide chain. The amino acid sequences of the α chains of pea (25, 26), lentil (27), and fava bean (28) lectins have been determined and show profound homology with one another and significant homology to the sequence of residues 70–119 of Con A, which has a single polypeptide chain subunit. The NH_2-terminal sequence of the β chains of all three lectins also display homology with one another and a correspondence to the COOH-terminal (residues 120–237) region of Con A (27, 29).

The use of lectin-Sepharose columns to assess the carbohydrate-binding specificity of pea and lentil lectin is very convenient and has the advantage of providing the basis for an effective method of fractionating glycopeptide mixtures with quantitative recovery of the applied radioactive glycopeptides. However, it must be kept in mind that this technique only measures relative affinities, and the behavior of glycopeptides may vary depending on the density of lectin coupled to the Sepharose or the size of the column used. Baenziger and Fiete (9) have determined the association constants of a series of glycopeptides for binding to Con A and also measured their ability to bind to Con A-Sepharose. In their series of 26 different complex type glycopeptide structures, they found that those with association constants between 4.5 and 25 × 10^6 M^{-1} bound to Con A-Sepharose, but those with association constants between 0.3 and 4.0 × 10^6 M^{-1} could not bind. This threshold effect for tight binding undoubtedly occurs with the pea and lentil lectin-Sepharose columns used in this study and accounts for the lack of binding by the high mannose type glycopeptides, which other studies indicate have affinity, albeit weak, for pea and lentil lectins. In this connection, it may be noted that it is often possible to retard weakly interacting

glycopeptides on columns of lectin-Sepharose if the columns are very long and the amount of material loaded is small compared to the amount of immobilized lectin. This is precisely the situation exploited by us (see under "Experimental Procedures") to fractionate the IgG glycopeptides on RCAI-Sepharose into those with 0, 1, and 2 terminal galactose residues using only buffer elution. It has been found (15) that complex glycopeptides must have 3 terminal β-galactose residues exposed in order to bind tightly enough to RCAI-Sepharose to require lactose for elution.

Because of their differences from Con A-Sepharose in carbohydrate-binding specificity, pea and lentil lectin-Sepharose offer useful tools for fractionation of glycopeptide mixtures unresolved by prior fractionation on Con A-Sepharose. Thus, complex type glycopeptides which bind to Con A-Sepharose can be further separated on pea lectin-Sepharose into those containing fucose, which bind, and those without fucose, which do not bind. The ability of pea and lentil lectin-Sepharose to interact with triantennary complex glycopeptides containing a mannose residue substituted at C-2 and C-6 has allowed us to separate a glycopeptide with this structure from the mixture of mouse lymphoma cell glycopeptides that do not bind to Con A-Sepharose. A glycopeptide with the same characteristics could also be isolated from calf thymocyte membrane glycopeptides, Madin-Darby bovine kidney cell glycopeptides, and the glycopeptides from vesicular stomatitis virus-infected Chinese hamster ovary cells.

REFERENCES

1. Reitman, M. L., Trowbridge, I. S., and Kornfeld, S. (1980) *J. Biol. Chem.* **255,** 9900–9906
2. Trowbridge, I. S., Hyman, R., Ferson, T., and Mazauskas, C. (1978) *Eur. J. Immunol.* **8,** 716–723
3. Allen, A. K., Desai, N. N., and Neuberger, A. (1976) *Biochem. J.* **155,** 127–135
4. Van Wauwe, J. P., Loontiens, F. G., and DeBruyne, C. K. (1975) *Biochim. Biophys. Acta* **379,** 456–561
5. Kornfeld, S., Rogers, J., and Gregory, W. (1971) *J. Biol. Chem.* **246,** 6581–6586
6. Young, M. N., and Leon, M. A. (1974) *Biochim. Biophys. Acta* **365,** 418–424
7. Toyoshima, S., Fukuda, M., and Osawa, T. (1972) *Biochemistry* **11,** 4000–4005
8. Kornfeld, R., and Ferris, C. (1975) *J. Biol. Chem.* **250,** 2614–2619
9. Baenziger, J. U., and Fiete, D. (1979) *J. Biol. Chem.* **254,** 2400–2407
10. Adair, W. L., and Kornfeld, S. (1974) *J. Biol. Chem.* **249,** 4696–4704
11. Trowbridge, I. S., Nilsen-Hamilton, M., Hamilton, R. T., and Bevan, M. J. (1977) *Biochem. J.* **163,** 211–217
12. Li, Y. T., and Li, S. C. (1972) *Methods Enzymol.* **28B,** 702–713
13. Kornfeld, R., Keller, J., Baenziger, J., and Kornfeld, S. (1971) *J. Biol. Chem.* **246,** 3259–3268
14. Baenziger, J., and Kornfeld, S. (1974) *J. Biol. Chem.* **249,** 7270–7281
15. Baenziger, J. U., and Fiete, D. (1979) *J. Biol. Chem.* **254,** 9795–9799
16. Brenckle, R., and Kornfeld, R. (1980) *Arch. Biochem. Biophys.* **201,** 160–173
17. Li, E., and Kornfeld, S. (1979) *J. Biol. Chem.* **254,** 1600–1605
18. Kornfeld, S., Gregory, W., and Chapman, A. (1979) *J. Biol. Chem.* **254,** 11649–11654
19. Beyer, T. A., Rearick, J. I., Paulson, J. C., Prieels, J. P., Sadler, J. E., and Hill, R. L. (1979) *J. Biol. Chem.* **254,** 12531–12541
20. Hakomori, S. (1964) *J. Biochem. (Tokyo)* **55,** 205–208
21. Li, E., Tabas, I., and Kornfeld, S. (1978) *J. Biol. Chem.* **253,** 7762–7770
22. Trevelyan, W. E., Proctor, D. P., and Harrison, J. S. (1950) *Nature (Lond.)* **166,** 444–445
23. Ogata, S., Muramatsu, T., and Kobata, A. (1975) *J. Biochem. (Tokyo)* **78,** 687–696
24. Hickman, S., Kornfeld, R., Osterland, K., and Kornfeld, S. (1972) *J. Biol. Chem.* **247,** 2156–2163
25. Van Driessche, E., Foriers, A., Strosberg, A. D., and Kanarek, K. (1976) *FEBS Lett.* **71,** 220–222
26. Richardson, C., Behnke, W. D., Freisheim, J. H., and Blumenthal, K. M. (1978) *Biochim. Biophys. Acta* **537,** 310–319
27. Foriers, A., DeNeve, R., Kanarek, L., and Strosberg, A. D. (1978) *Proc. Natl. Acad. Sci. U. S. A.* **75,** 1136–1139
28. Hemperly, J. J., Hopp, T. P., Becker, J. W., and Cunningham, B. A. (1979) *J. Biol. Chem.* **254,** 6803–6810
29. Cunningham, B. A., Hemperly, J. J., Hopp, T. P., and Edelman, G. M. (1979) *Proc. Natl. Acad. Sci. U. S. A.* **76,** 3218–3222