THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 258, No. 1, Issue of January 10, pp. 199-202, 1983
Printed in U.S.A.

Binding of Synthetic Oligosaccharides to the Hepatic Gal/GalNAc Lectin

DEPENDENCE ON FINE STRUCTURAL FEATURES*

(Received for publication, July 20, 1982)

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A series of synthetic oligosaccharides, resembling natural *N*-acetyllactosamine type glycans, were tested for their ability to inhibit the binding of labeled ligand to the mammalian hepatic lectin on rabbit hepatocytes at 2 °C. A dramatic hierarchy of inhibitory potency (tetraantennary > triantennary ≫ biantennary ≫ monoantennary) could be demonstrated. The range of concentration required for 50% inhibition of labeled ligand binding extended from ~1 mM, for the monoantennary oligosaccharides, to ~1 nM for triantennary oligosaccharides, even though the absolute Gal concentration increased only 3-fold. It was found that the number of Gal residues/cluster and their branching mode are major determinants of binding affinity of ligands to the hepatic lectin on the surface of hepatocytes.

It has been shown that the hepatic lectin on the surface of mammalian hepatocytes can effectively discriminate between clusters of 1, 2, and 3 galactose[1] residues. This has been demonstrated using either synthetic cluster glycosides (1) or asialoglycopeptides derived from glycoproteins (2). However, the estimated affinity of synthetic *tris*-galactosides (1) was found to be 1000-fold less than that of the naturally derived three-Gal cluster, asialotriantennary glycopeptide (2). A major difference between these two ligands is that in the triantennary type oligosaccharide chain, the galactose residues are farther apart from the branching point, separated by three to five monosaccharides, while in cluster glycosides the Gal residues are spaced by three carbons. In order to study the effect of positioning of terminal Gal residues, we tested the relative potency of a series of glycopeptides and oligosaccharides (shown in Diagrams 1, 2, and 3) to inhibit binding of labeled ligands to the Gal/GalNAc lectin of mammalian liver.

Since the "cluster effect" of Gal residues was demonstrated more distinctly with intact hepatic parenchymal cells (1, 2), inhibition of ligand binding to isolated rabbit hepatocytes was studied. The relative affinity of the oligosaccharides was estimated from the concentration of ligand required for 50% inhibition of binding ($[I]_{50}$) of ^{125}I-Tyr-asialotriantennary glycopeptide to rabbit hepatocytes at 2 °C. Inhibitory potency of the oligosaccharides was in the expected order of tetraantennary > triantennary > biantennary > monoantennary, and the effect of "clustering" was clearly demonstrated. For example, it was found that the inhibitory power of a tetraantennary ligand ($[I]_{50} = 10^{-9}$ M) was 1×10^6 greater than that of a monoantennary ligand ($[I]_{50} = 10^{-3}$ M) while the total galactose concentration was only 4-fold greater. In addition, within each group of the oligosaccharides, subtle changes in structural features (such as positions of branching) exerted great influence on the relative binding affinity.

EXPERIMENTAL PROCEDURES

Materials—Hepes[2] was obtained from Research Organics (Cleveland, OH). Silicone oil (DC 550) was obtained from Accumetric (Elizabethtown, KY) and light mineral oil from Barre Drug Co., Inc. (Baltimore, MD). (^{125}I)NaI (carrier-free) in 0.1 M NaOH was obtained from Amersham Corp. (Arlington Hts., IL). Pyridine-acetate buffer, pH 4.7, was prepared by mixing 25 ml of pyridine and 25 ml of acetic acid and made up to 1 liter of water. Sephadex G-50 and Concanavalin A-Sepharose were obtained from Pharmacia (Uppsala, Sweden). Bio-Gel P-4 was from Bio-Rad Laboratories (Richmond, CA). Pronase was purchased from Calbiochem (San Diego, CA). *Arthrobacter* neuraminidase was a gift from Dr. Y. Uchida (Kyoto Research Laboratories, Uji, Japan). A unit of neuraminidase activity is defined as the amount required to release 1 μmol of sialic acid from colominic acid (3). Leucine aminopeptidase from hog kidney and carboxypeptidase Y from yeast were from Boehringer-Mannheim (Indianapolis, IN). Alpha-1-protease inhibitor was a gift from Dr. R. Glew (Department of Biochemistry, University of Pittsburgh, PA).

Synthetic Oligosaccharides—The structures of synthetic oligosaccharides are shown in Diagrams 2 and 3. The synthesis of Galβ(1,4)GlcNAcβ(1,6)Man, HEPTA, PENTA-2,4, PENTA-2,6, and NONA I has been published (4–7). Other oligosaccharides were prepared by similar methods[3].

Asialoglycopeptides—Asialobiantennary glycopeptide (Diagram 1) was prepared from human fibrinogen as described (8). Triantennary

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§ To whom all correspondence should be addressed. Supported by United States Public Health Service-National Institutes of Health Research Grants AM 9970 and CA 21901.

|| Supported by United States Public Health Service-National Institutes of Health Training Grant HL 06188 and by the Daland Fund of the American Philosophical Society.

** Supported by a Fellowship from The Johns Hopkins University.

§§ Supported by the Swedish Natural Science Research Council.

[1] All sugars are of D-configuration in pyranose form, unless otherwise stated.

[2] The abbreviations used are: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Gal$_n$-AI-bovine serum albumin, β-D-galactopyranosylthioacetamidino-bovine serum albumin with *n* residues of Gal attached (Ref. 15). Abbreviations used for glycopeptides and synthetic oligosaccharides are given in Diagrams 1, 2, and 3.

[3] J. Lönngren, J. Arnarp, M. Haraldsson, and H. Lönn, unpublished results.

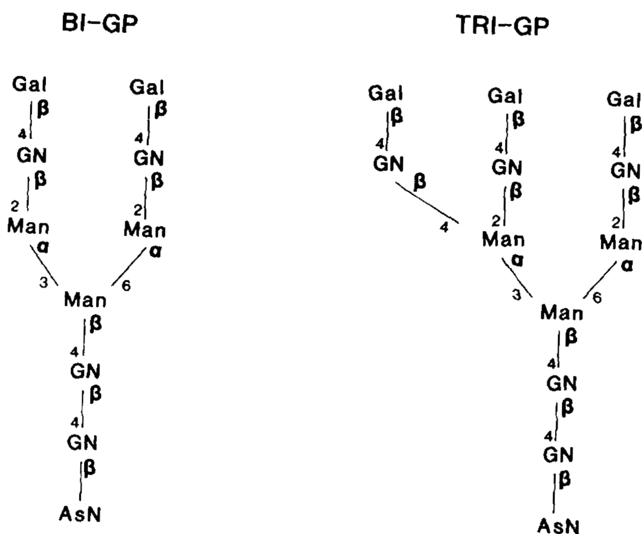

DIAGRAM 1

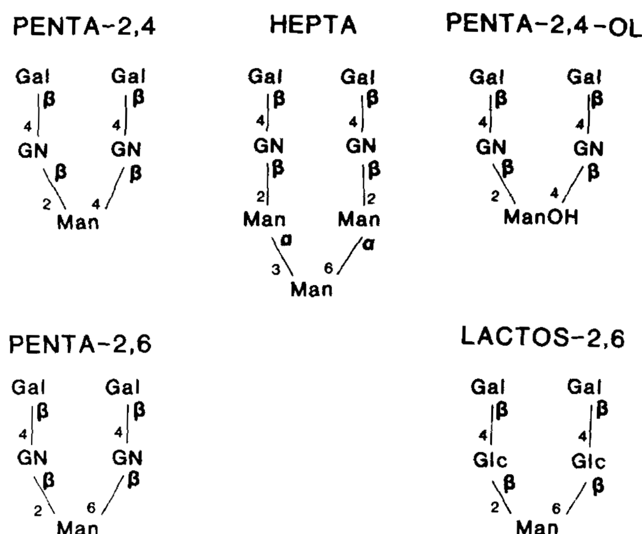

DIAGRAM 2

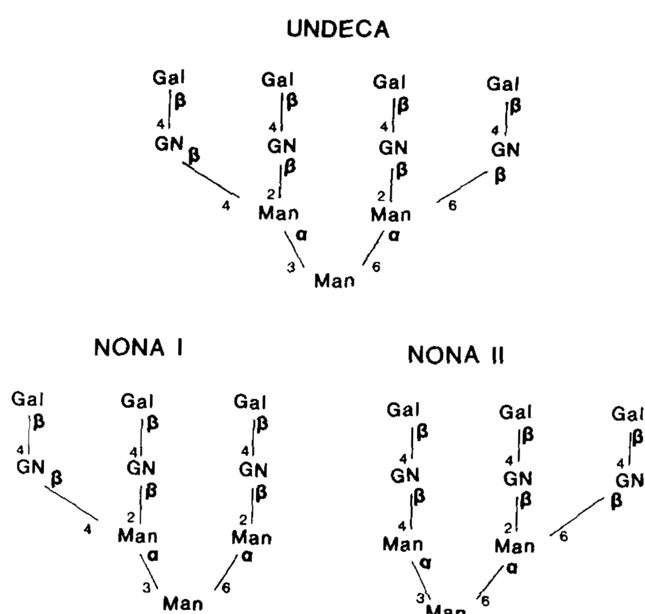

DIAGRAM 3

glycopeptide (Diagram 1)[4] was prepared from α-1-protease inhibitor by a modification of the procedure previously described (9). Briefly, the glycoprotein (1 g) was subjected to 2 cycles of pronase digestion with isolation of the glycopeptides after each step. The glycopeptides were treated with 0.1 unit of neuraminidase. The release of sialic acid was complete after 24 h, as measured by a modification of the thiobarbituric acid method (3). Further peptide digestion was accomplished by treating the desialylated glycopeptides with 1 unit of leucine aminopeptidase and 1 unit of carboxypeptidase Y for 2 days at 37 °C in 0.05 M Hepes buffer, pH 6.0. The digest was applied to a Bio-Gel P-4 column (5 × 100 cm) equilibrated in pyridine-acetate buffer, pH 4.7, and analyzed for amino groups (10) and carbohydrate (11). Two peaks, both containing amino groups and carbohydrate, were pooled separately. The front peak, enriched in triantennary glycopeptides, was lyophilized and subjected to Concanavalin A-Sepharose chromatography to separate biantennary from triantennary glycopeptides (9). The material not binding to the Concanavalin A-Sepharose was applied to the Bio-Gel P-4 column, as described above, for further purification. Tyrosine was coupled to asialotriantennary glycopeptide as previously described (1), and the Tyr-asialotriantennary glycopeptide was iodinated by a modified chloramine-T method (12) using a 2-fold molar excess of [^{125}I]NaI over glycopeptide. The resulting ^{125}I-Tyr-asialotriantennary glycopeptide had a specific activity of approximately 1×10^9 cpm/nmol.

Inhibition Assay—Stock solutions of inhibitors were prepared by dissolving dried oligosaccharides or asialoglycopeptides in water. The exact concentration was determined by analysis of component neutral sugars by automated liquid chromatography (13). The stock solutions were appropriately diluted with modified Dulbecco's Eagle's medium (14). The assays were carried out at 2 °C in capped polystyrene tubes (12 × 75 mm). Each tube contained approximately 2.5×10^6 rabbit hepatocytes, prepared by an established procedure,[5] inhibitor of known concentration, and 0.8 pmol of ^{125}I-Tyr-asialotriantennary glycopeptide in a total volume of 1 ml of modified Dulbecco's Eagle's medium. In some experiments, ^{125}I-asialoorosomucoid (2.5×10^{-10} M) was used as the labeled ligand. The tubes were rotated vertically at 4 rpm for 2 h at 2 °C, after which time duplicate 200-μl samples were taken and pipetted into 400-μl microfuge tubes containing approximately 150 μl of silicone/mineral oil mixture (4:1, v/v) at 2 °C. The tubes were immediately centrifuged in an Eppendorf microfuge (Model 5412) for 10 s. The bottom of the tube, containing the cell pellet, was snipped off and counted for radioactivity in a Packard PRIAS auto γ counter. Nonspecific binding was defined as the amount of ^{125}I-Tyr-asialotriantennary glycopeptide bound to the cells when the incubation was carried out in the presence of a 2000-fold molar excess of Gal_{44}-AI-bovine serum albumin (15). The $[I]_{50}$ was determined using a computer curve-fitting program (ALLFIT) based on a logistic equation as previously described (16). The ALLFIT program was obtained from Biomedical Computer Technology Information Center (Vanderbilt Medical Center, Nashville, TN). The program was implemented on a DEC-10 computer at The Johns Hopkins University Computing Center.

RESULTS AND DISCUSSION

It is now apparent that the number and orientation of Gal residues on the ligand are major determinants of affinity for the hepatic Gal/GalNAc receptor. Affinity increased exponentially with the number of Gal residues coupled to bovine serum albumin suggesting that clustering of sugar residues may be important in receptor binding (17, 18). This "cluster effect" was demonstrable in the binding of synthetic cluster galactosides to isolated hepatocytes, but not to the isolated lectin (1). A similar trend was reported for the isolated human hepatic lectin using asialobi- and asialotriantennary glycopeptides in which the K_i values were 3.9×10^{-5} and 1×10^{-6} M,

[4] The structure shown in Diagram 1 for the triantennary glycopeptide depicts one of the two possible branching patterns (22). The other reported structure places the third β-N-acetyllactosaminyl unit on the 6-linked Man rather than on the 3-linked Man (23).

[5] D. T. Connolly, R. R. Townsend, K. Kawaguchi, M. K. Hobish, W. R. Bell, and Y. C. Lee, manuscript in preparation.

respectively (19). However, the K_d reported for asialotriantennary glycopeptide from studies with isolated rat hepatocytes was 1×10^{-8} M (2), a 100-fold difference relative to the isolated lectin. Therefore, the binding properties of the lectin in hepatocytes were characterized in this study.

We present data on the relative contribution of the composition of oligosaccharide branches and their mode of branching to ligand affinity. This was accomplished with synthetic oligosaccharides which resemble naturally occurring *N*-acetyllactosamine type oligosaccharides. They displayed a dramatic hierarchy (tetra- > tri- ≫ bi- ≫ mono-) of inhibitory potencies. As shown in Fig. 1 and Table I, the $[I]_{50}$ values for mono-, bi-, and triantennary oligosaccharides were found to be approximately 1×10^{-3}, 1×10^{-6}, and 5×10^{-9} M, respectively. In other words, though the number of Gal residues/mol of ligand increased only 3-fold, the inhibitory potency increased nearly 1,000,000-fold.

The monoantennary ligand, Galβ(1,4)GlcNAcβ(1,6)Man, had a similar apparent affinity as Gal, approximately 1 mM. However, addition of another *N*-acetyllactosamine chain to

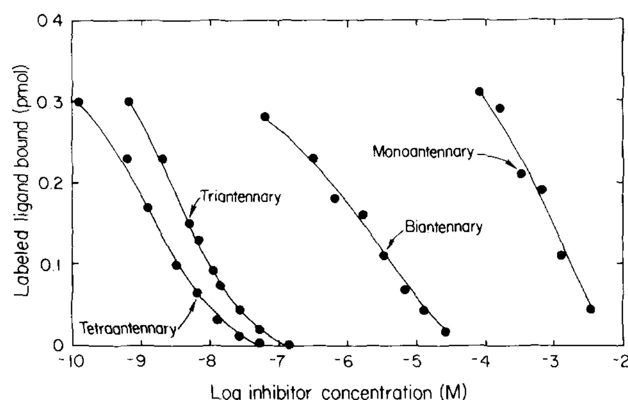

FIG. 1. **Inhibition of binding of ^{125}I-Tyr-asialotriantennary glycopeptide to rabbit hepatocytes at 2 °C.** Unlabeled ligands were incubated with freshly isolated hepatocytes at the indicated concentrations. The monoantennary ligand used was Galβ(1,4)GlcNAcβ(1,6)Man. Asialobiantennary glycopeptide from human fibrinogen was used as the biantennary ligand. The triantennary ligand used was the asialotriantennary glycopeptide from human α-1-protease inhibitor. UNDECA was the tetraantennary ligand. Details of the preparation of these ligands and inhibition assay are given under "Experimental Procedures."

TABLE I
Comparison of $[I]_{50}$ using either ^{125}I-asialoorosomucoid or ^{125}I-Tyr-tri-GP as labeled ligand

Compounds	$[I]_{50}$	
	Asialoorosomucoid[a]	TYR-TRI-GP[b]
	μM	
GalGlcNAcMan	ND[c]	821 ± 242
HEPTA	49.9 ± 15.7	20.4 ± 1.5
LACTOS-2,6	6.2 ± 2.6	3.6 ± 0.37
PENTA-2,6	4.5 ± 1.4	2.8 ± 0.21
PENTA-2,4-OL	1.5 ± 0.64	1.9 ± 0.17
PENTA-2,4	0.27 ± 0.11	0.25 ± 0.04
BI-GP	2.4 ± 0.95	1.8 ± 0.20
	nM	
NONA II	145 ± 40.6	111 ± 11.2
TYR-TRI-GP	ND	6.2 ± 0.59
NONA I	7.4 ± 2.3	2.0 ± 0.34
UNDECA	3.4 ± 1.0	1.3 ± 0.14
TRI-GP	5.8 ± 1.9	4.3 ± 0.48

[a] ^{125}I-asialoorosomucoid (2.5×10^{-10} M) was used as labeled ligand.
[b] ^{125}I-Tyr-TRI-GP (8×10^{-10} M) was used as labeled ligand.
[c] ND, not determined.

the terminal Man (to form a biantennary structure) resulted in ligands with $[I]_{50}$ values ranging from 10^{-5} to 10^{-7} M (Table I). The inhibition curves for ligands of biantennary structure are shown in Fig. 2. Desialylated BI-GP, and some synthetic analogs, PENTA-2,6 and LACTOS-2,6, had similar $[I]_{50}$ values, about 10^{-6} M. In comparing the structures of two synthetic pentasaccharides, PENTA-2,6 and LACTOS-2,6, it is apparent that the replacement of GlcNAc with Glc in both branches did not change the binding affinity. Interestingly, these two structures had the same affinity as desialylated biantennary glycopeptide in which each of the β-*N*-acetyllactosamine chains are spaced by an additional Man from the branching Man (Diagram 2). On the other hand, a synthetic heptasaccharide (HEPTA) corresponding to the same structure as found in the biantennary glycopeptide, except for the absence of the GlcNAcβ(1,4)GlcNAcβAsn, gave an unexpected result. The heptasaccharide was found to be less potent an inhibitor, having a 20-fold higher $[I]_{50}$ than asialobiantennary glycopeptide. Similarly, biantennary glycopeptide binding to the red kidney bean lectin (*Phaseolus vulgaris*) was shown to be 4-fold better than the heptasaccharide (20).

PENTA-2,6 and LACTOS-2,6, having two branches β-linked to Man at the 2 and 6 positions, showed similar affinities toward the lectin as the asialobiantennary glycopeptide. However, placement of β-*N*-acetyllactosaminyl units at positions 2 and 4 of the Man (PENTA-2,4) resulted in a ligand with 10-fold lower $[I]_{50}$ than asialobiantennary glycopeptide. It should be noted that this pentasaccharide unit is a part of the natural triantennary glycopeptide (Diagram 1), and thus may be a major structural determinant in the tighter binding ($[I]_{50}$ = 4.3×10^{-9} M) displayed by the triantennary glycopeptide. Reduction of the terminal Man (PENTA-2,4-ol) is accompanied by a 5-fold increase in the $[I]_{50}$ (Fig. 2 and Table I) suggesting that branching from an acyclic mannitol resulted in changes in conformation.[6] It is interesting to note that in the most favorable conformation, the Gal residues in PENTA-2,4 appear to be separated in space by ~15 Å while those in PENTA-2,6 and HEPTA by ~23 (or ~15) and ~25 Å, respectively.[6]

Fig. 3 shows inhibition curves for triantennary and tetraantennary ligands. NONA I possesses a structure similar to the one reported for triantennary oligosaccharides isolated from α-1-acid glycoprotein (21) and fetuin (22). The $[I]_{50}$ was similar

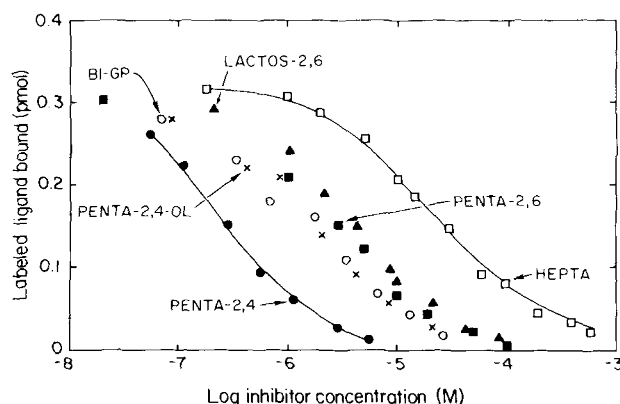

FIG. 2. **Inhibition of binding of ^{125}I-Tyr-asialotriantennary glycopeptide to rabbit hepatocytes at 2 °C by biantennary ligands.** Unlabeled ligands were incubated with freshly isolated hepatocytes at the indicated concentrations. Details of preparation of oligosaccharides and inhibition assay are given under "Experimental Procedures." Complete structures of ligands are shown in Diagrams 1 and 2.

[6] K. Bock, and J. Lönngren, manuscript in preparation.

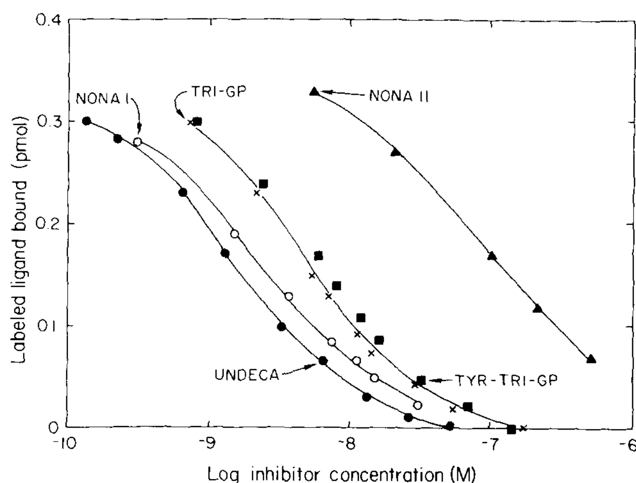

FIG. 3. **Inhibition of binding of ^{125}I-Tyr-asialotriantennary glycopeptide to rabbit hepatocytes at 2 °C by triantennary and tetraantennary ligands.** Unlabeled ligands were incubated with freshly isolated hepatocytes at the indicated concentrations. Details of preparation of oligosaccharides and inhibition assays are given under "Experimental Procedures." Complete structures of ligands are shown in Diagrams 1 and 3. *TYR-TRI-GP* is asialotriantennary glycopeptide from α-1-protease inhibitor (Diagram 1) after coupling of *Tyr* as described under "Experimental Procedures."

to the value found for asialotriantennary oligosaccharide from α-1-protease inhibitor.

The structure of NONA II is different from that of NONA I in the attachment positions of the β-*N*-acetyllactosamine chains and resembles one of the branching patterns reported for the triantennary from fetuin (23). It is puzzling that NONA II possesses an $[I]_{50}$ of around 100 nM when it was reported that the K_d for asialotriantennary glycopeptides from fetuin is 10 nM (2). Although NONA II is much less inhibitory than NONA I, it is still 40-fold more inhibitory than PENTA-2,6, which is a part of NONA II.

Undecasaccharide, UNDECA, possesses four lactosamine branches representing the partial structure of the tetraantennary glycopeptides isolated from human α-1-acid glycoprotein (21). It was found to have only a slightly higher affinity for the lectin than NONA I or asialotriantennary glycopeptide from α-1-protease inhibitor. An additional β-*N*-acetyllactosamine 6-linked to the branching Man in NONA I does not markedly enhance the $[I]_{50}$ of that ligand. It is possible that the binding requirement of the cell-surface receptors is largely satisfied by the triantennary structure. However, in view of the difference in inhibitory potency between NONA I and NONA II, it is also possible that tetraantennary oligosaccharides of different structural design may show greater increase in binding affinity.

The Gal-Gal distance deduced from the optimal conformational structures of these oligosaccharides generally follows the hierarchy of the inhibitory potency. Although it was speculated that 25-30 Å between Gal/GalNAc residues may provide optimal binding (19), our results indicate the Gal-Gal distance of ~15 Å produced nearly 100-fold stronger binding than those with the distance of 25 Å.

In a similar study on binding of synthetic and natural oligosaccharides by the *P. vulgaris* lectin (20), different binding specificity was noted. It was found that asialofetuin glycopeptide (mostly triantennary) was only as effective as PENTA-2,6, and PENTA-2,4 was inferior to PENTA-2,6.

Our studies showed that the number of Gal residues/cluster and their branching mode (and consequently the distance between the Gal residues) are major determinants of binding affinity of the ligand to the mammalian hepatic lectin on the surface of hepatocytes. The importance of which mannose residue bears the additional lactosamine branch in the triantennary structure was emphasized by the 50-fold lower $[I]_{50}$ for NONA I compared to NONA II. In addition it was found that the branch sugars internal to the Gal residues could be changed without significantly reducing the binding affinity, at least in the case of biantennary ligands. How the lectin on the hepatocyte surface discriminates the fine structural features of oligosaccharides and glycosides is an interesting question. Whether this is a property of a single lectin molecule or is related to aggregation of lectin molecules on the cell surface merits further studies.

Acknowledgments—We would like to thank Theresa Kuhlenschmidt for generously supplying Gal_{44}-AI-bovine serum albumin. The help with laboratory facilities given by Dr. W. R. Bell is greatly appreciated.

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