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Carbohydrate Binding Studies on the Lectin from *Datura stramonium* Seeds[1]

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The carbohydrate-binding properties of the *Datura stramonium* seed lectin were studied by equilibrium dialysis, quantitative precipitation of natural and synthetic glycoproteins, and hapten inhibition of precipitation. The dimeric lectin (M_r = 86,000) possesses two carbohydrate-binding sites for *N,N',N'',N'''*-tetraacetylchitotetritol/mol protein, with an apparent $K_a = 8.7 \times 10^3$ M^{-1} at 4°C. Whereas fetuin and orosomucoid reacted poorly with the *Datura* lectin, the asialo derivatives of these glycoproteins gave strong precipitation with the lectin. Carcinoembryonic antigen, type 14 pneumococcal capsular polysaccharide, and bovine serum albumin, highly substituted with *N,N'*-diacetylchitobiose units, also precipitated the lectin. Of the homologous series of chitin oligosaccharides tested, *N,N',N'''*-triacetylchitotriose was over 6-fold more potent than the disaccharide (*N,N'*-diacetylchitobiose) which, in turn, was 90 times more reactive than *N*-acetyl-D-glucosamine. *N*-Acetyllactosamine [β-D-Gal-(1 → 4)-D-GlcNAc] was also a potent inhibitor of Datura lectin being equivalent to *N,N'*-diacetylchitobiose. The requirement for an *N*-acetyl-D-glucosaminyl unit linked at the C-4 position was established. The biantennary pentasaccharide (penta-2,6) was a 500-fold more potent inhibitor than *N*-acetyllactosamine, suggesting that it might interact with both saccharide-binding sites of the *Datura* lectin simultaneously.

The seeds of *Datura stramonium* (jimson weed or thorn apple) contain a lectin which specifically binds β-(1 → 4)-linked oligomers of *N*-acetyl-D-glucosamine (1, 2). It has been partially purified by affinity chromatography on fetuin–Sepharose (3), ovomucoid–Sepharose (4), glutaraldehyde-fixed erythrocytes (5, 6), and on an insoluble polysaccharide mixture from *Aspergillus niger* mycelia (7). A recent report from this laboratory (8) described the purification of the *Datura* lectin on an *N,N'*-diacetyl-β-chitobioside–Sepharose column. The purified lectin appeared homogeneous by sedimentation analysis, gel filtration, electrophoresis under both native and denaturing conditions, and immunoelectrophoresis; the 32,000-d protein which has contaminated all previously reported lectin preparations was not detected. The *Datura* lectin, a dimeric glycoprotein composed of two nonidentical subunits (M_r 40,000 and 46,000) joined by disulfide bonds (3, 4, 8, 9), contains 37% carbohydrate by weight, of which 93% is arabinose and 7% is galactose (4, 8, 9).

In this communication, we report on the carbohydrate-binding specificity of *Datura* lectin. The physicochemical and biological properties of the lectin will be described in a separate communication.

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MATERIALS AND METHODS

Sugars and sugar derivatives. *p*-Nitrophenyl *N,N'*-diacetyl-β-chitobioside and *p*-nitrophenyl *N,N',N''*-triacetyl-β-chitotrioside were synthesized by the method of Osawa (10). *p*-Nitrophenyl-α-D-GlcNAc*p*[3] was purchased from Vega Biochemicals, Tucson, Arizona; *p*-nitrophenyl-β-D-Gal*p* was purchased from Koch-Light Laboratories, Colnbrook Bucks, England. *p*-Nitrophenyl β-D-GlcNAc*p* was available in this laboratory from previous studies. Per-*N*-acetylated chitobiose and chitotriose were prepared in this laboratory by acetolysis of chitin (11) by Dr. R. Kaifu. The corresponding 1-chloro- and β-methyl- derivatives were synthesized according to the method of Dahlquist and Raftery (12). Methyl β-chitobioside octaacetate was *O*- and *N*-deacetylated by treatment with anhydrous hydrazine at 100°C for 72 h (13). The chitobioside was reacted with *S*-ethyltrifluorothioacetate (14) to afford methyl *N,N'*-ditrifluoroacetyl-β-chitobioside. Reaction of the chitobioside with propionic anhydride (Kaifu *et al.*, submitted) gave methyl *N,N'*-dipropionyl-β-chitobioside. Heptaacetylchitobiose hydrochloride was synthesized as described by Dahlquist *et al.* (12), and de-*O*-acetylated with sodium methoxide to give β-D-GlcNAc-(1 → 4)-D-GlcN. Treatment of this disaccharide with *S*-ethyltrifluorothioacetate or propionic anhydride, as described above, gave *N*-trifluoroacetyl, *N'*-acetylchitobiose, and *N*-propionyl, *N'*-acetylchitobiose, respectively. Reaction of heptaacetylchitobiose hydrochloride with benzoyl chloride in pyridine (Kaifu *et al.*, submitted), followed by de-*O*-acetylation, gave *N*-benzoyl, *N'*-acetylchitobiose. Dr. R. Spiro, Harvard University, donated β-D-Man-(1 → 4)-D-GlcNAc and β-D-GlcNAc-(1 → 6)-D-Gal. Dr. G. W. Jourdian of this University provided β-D-GlcNAc(1 → *N*)Asn. The following synthetic oligosaccharides, the structures of which are shown in Fig. 4, were provided by Arnarp and Lönngren (15-18): hepta; penta-2,6; penta-2,4; tri-2,6; tri-2,4; β-D-Gal-(1 → 4)-β-D-GlcNAc-(1 → 2)-D-Man; β-D-Gal-(1 → 4)-β-D-GlcNAc-(1 → 6)-D-Man; nona I; nona II; undeca; Hexa-3,6; and aryl penta-2,6. *N*-Acetyllactosamine [β-D-Gal-(1 → 4)-D-GlcNAc], β-D-GlcNAc-(1 → 6)-D-Gal, 4-*O*-methyl-D-GlcNAc, and methyl 3-*O*-methylβ-D-GlcNAc*p* were synthesized by Nike Plessas of this laboratory. Methylβ-D-GlcNAc*p* and 3,6-di-*O*-(β-D-GlcNAc*p*)-D-Gal*p* were available in this laboratory from previous studies.

Glycoproteins and polysaccharides. *D. stramonium* seeds were obtained from the Matthaei Botanical Gardens, University of Michigan. *Datura* lectin was purified according to the method described previously (8). Carcinoembryonic antigen, S-14 pneumococcal polysaccharide, and orosomucoid were available in this laboratory from previous studies. Fetuin was purchased from Gibco, Grand Island, New York.

Sialic acid was selectively removed from fetuin and orosomucoid by treatment at 80°C for 1 h in 0.1 N sulfuric acid (19). Desialation was determined using the thiobarbituric acid assay of Warren (20). The pentultimate galactose residues was removed by treatment of the asialoglycoprotein with bovine testicular β-galactosidase, prepared by Diane Blake in this laboratory according to the method of Distler *et al.*, (21). The glycoprotein was incubated with β-galactosidase (1/2 unit/ml reaction volume) in 0.1 M citrate buffer, pH 4.5, for 6 h at 37°C. An aliquot of the reaction mixture was analyzed for free galactose using a galactose dehydrogenase assay (22). The β-galactosidase was removed by applying the reaction mixture to an affinity matrix of agarose-β-1-thiogalactoside obtained from Dr. Jourdian of this Department. The β-galactosidase binds to the matrix at pH 5, and can be eluted at pH 7.5.

Precipitin and hapten inhibition reactions. Precipitin reactions were conducted as described by So and Goldstein (23) using 27 μg *Datura* lectin and varying amounts of glycoconjugate per tube. Total volume was brought to 0.5 ml with PBS. Sugar inhibition of the precipitin reaction was conducted by adding increasing amounts of carbohydrate hapten to the tubes containing *Datura* lectin (27 μg) in PBS. After a 10-min preincubation period, the precipitin reaction was initiated by addition of asialofetuin (80 μg) to give a final volume of 0.5 ml. The inhibition curve of *N,N'*-diacetylchitobiose was routinely run as a standard.

Equilibrium dialysis. Radioactive ligand was obtained by reduction of *N,N',N'',N'''*-tetraacetylchitotetraose with [^{3}H]borohydride. Equilibrium dialysis experiments were performed in duplicate in a multichambered dialysis cell (Scientific Specialties, Inc., Randallstown, Md.). Lectin concentration was 21 μM and initial ligand concentrations varied from 50 μM to 1 mM. Filled dialysis cells, each containing 350 μl solution, were slowly rotated for 48 h at 4°C. Upon reaching equilibrium, aliquots (40 μl) were removed and counted in ACS counting scintillant (Amersham Corp., Arlington Heights, Ill.). It was necessary to add an additional 200 μl water to each scintillation vial prior to counting in order to stabilize the counts.

RESULTS

A Scatchard plot of the binding of [^{3}H]*N,N',N'',N'''*-tetraacetylchitotetritol to *Datura* lectin at 4°C is shown in Fig. 1. The absence of curvature in the plot suggests that the sugar-binding sites are noninteracting and homogeneous with respect to association constant. Assuming a mo-

[3] Abbreviations used: GlcNAc*p*, N-acetylglucosamine pyranose; GlcN, glucosamine; PBS, phosphate-buffered saline; BSA, bovine serum albumin.

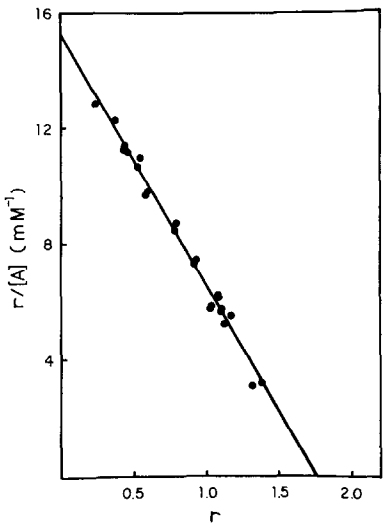


FIG. 1. Binding of [^{3}H]-*N,N',N'',N'''*-tetraacetylchitotetritol to *Datura* lectin plotted according to Scatchard. [A], concentration of free sugar; r, moles of sugar bound per mole lectin. Lectin concentration was 21 μM and initial ligand concentrations varied from 50 μM to 1 mM. Binding was performed at 4°C for 48 h.

lecular weight of 86,000, these data indicate that the dimeric lectin can bind 2 mol of sugar per mol protein, with an apparent association constant of 8.7×10^3 M^{-1}.

A number of glycoproteins and glycoconjugates containing terminal and/or internal *N*-acetyl-D-glucosamine residues were examined for their ability to form a precipitate with the *Datura* lectin. The lectin did not precipitate any synthetic glycoconjugates in which simple monosaccharides had been linked to bovine serum albumin (BSA). It did react with *p*-azophenyl *N,N'*-diacetyl-β-chitobioside–BSA, but only if the degree of substitution on BSA was high. A sample containing 5 disaccharide units per mol BSA was not precipitated by *Datura* lectin, whereas a sample with 17 residues per mol BSA did form a precipitate. By contrast, wheat germ agglutinin and potato lectin were able to precipitate both samples. Shier's antigen A (24), which contains *N,N'*-diacetylchitobiose glycosidically linked to poly-L-asparatate via asparaginyl bonds, did not precipitate the *Datura* lectin, presumably because of the low degree of substitution (13%). Other biopolymers found to be nonreactive with *Datura* lectin include ethylene glycol chitin, a soluble derivative of chitin in which some of the C-6 hydroxyl groups had been modified to the corresponding hydroxyethyl ethers; keratan sulfate, which is composed of repeating units of 3)-β-D-galactosyl-(1 → 4)-β-*N*-acetyl-D-glucosamine-6-sulfate-(1 →; and β-D-GlcNAc-(1 → 6)-D-Gal-BSA (21 residues/mol BSA).

Several glycoproteins containing internal *N*-acetyl-D-glucosamine residues were shown to precipitate with *Datura* lectin. rcinoembryonic antigen (CEA), a tumor-associated antigen composed of 50% carbohydrate of which a high proportion are β-(1 → 4) linked *N*-acetyl-D-glucosamine residues (25), reacted strongly with *Datura* lectin. Pneumococcus type 14 capsular polysaccharide, which consists of tetrasaccharide repeating units of the form (26),

$$\rightarrow 6)\text{-}\beta\text{-D-GlcNAc}p\text{-}(1 \rightarrow 3)\text{-}\beta\text{-D-Gal}p\text{-}(1 \rightarrow$$
$$\begin{matrix} 4 \\ \uparrow\beta \\ 1 \\ \text{Gal}p \end{matrix}$$
$$4)\text{-}\beta\text{-D-Glc}p\text{-}(1\rightarrow$$

also reacted strongly with *Datura* lectin.

The reactivity of *Datura* lectin with fetuin and orosomucoid, and their asialo and asialoagalacto analogs, is shown in Fig. 2. Fetuin and orosomucoid were subjected to mild acid hydrolysis, resulting in liberation of 92 and 93% of the sialic acid, respectively. Subsequent treatment of the asialoproteins with bovine testicular β-galactosidase resulted in removal of 93 and 78% of the galactosyl residues from fetuin and orosomucoid, respectively. *Datura* lectin reacted only weakly with both fetuin and orosomucoid. Desialation of the parent glycoproteins rendered them much more reactive towards precipitation with *Datura* lectin. It is apparent from these results that the presence of sialic acid interferes with the precipitation. This could be due to steric interference and/or nonspecific charge effects.

Removal of the galactosyl residues from asialofetuin resulted in a 50% decrease in

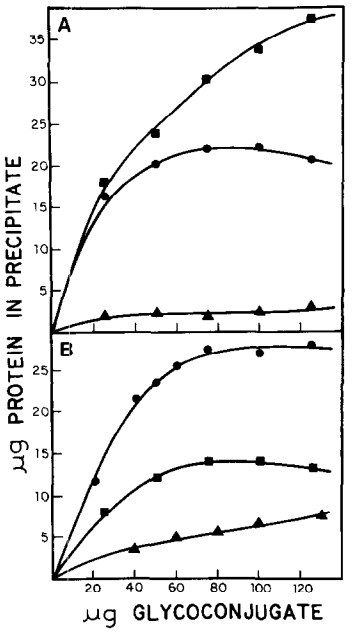


FIG. 2. Quantitative precipitation of fetuin, orosomucoid (OSM), and their analogs by *Datura* lectin. Each tube contained 27 μg lectin and increasing amounts of glycoprotein in a total volume of 500 μl. (A) OSM (▲); asialo-OSM (●); asialo-agalacto-OSM (■); (B) fetuin (▲); asialofetuin (●); asialo-agalactofetuin (■).

precipitated protein. Removal of the galactosyl residues from asialoorosomucoid, however, increased the reactivity towards *Datura* lectin (Fig. 2). The reason for the differential reactivity of the asialoagalacto-glycoproteins towards *Datura* lectin is uncertain. Although both fetuin and orosomucoid are *N*-acetyllactosamine-type glycans, there are structural differences in their carbohydrate moieties. It may be that *Datura* lectin can bind terminal, nonreducing *N*-acetyl-D-glucosamine residues only if there is a high density of such residues near the binding sites, as has been suggested by Debray *et al.* (27). The presence of L-fucose in the carbohydrate structure of orosomucoid may also play a role in the interactions with *Datura* lectin.

Inhibition of Datura Lectin–Asialofetuin Interaction by Low-Molecular-Weight Sugars

The carbohydrate-binding specificity of the *Datura* lectin was probed by sugar inhibition of the lectin–asialofetuin precipitation system. When plotted as percentage inhibition versus the inhibitor concentration on a logarithmic scale, typical sigmoidal inhibition curves were obtained (Fig. 3). The inhibitors tested, and the concentrations required for 50% inhibition by each hapten, are listed in Tables I and II.

As was originally noted by Pardoe *et al.* (2), *Datura* lectin displays a general spec-

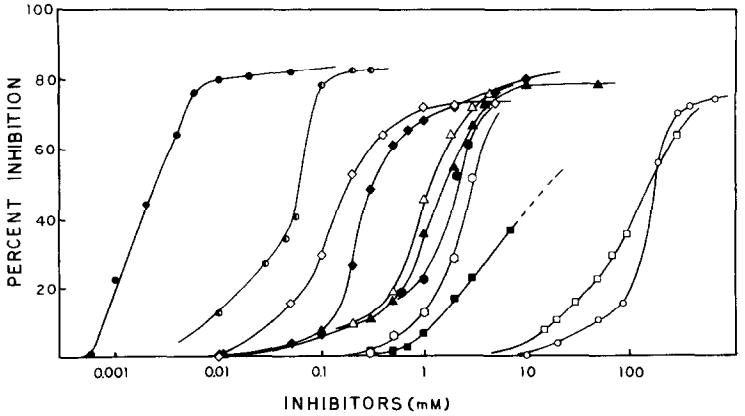


FIG. 3. Inhibition of *Datura* lectin–asialofetuin precipitation reaction by low-molecular-weight sugars. Conditions are stated under Material and Methods. ●, Penta-2,6; ◐, Penta-2,4; ◇, *N,N',N'',N'''*-tetraacetylchitotetraose; ◆, *N,N',N''*-triacetylchitotriose; △, *N*-acetyllactosamine; ▲, *N,N'*-diacetylchitobiose; ●, β-Gal-(1 → 4)-β-GlcNAc-(1 → 6)-Man; ○, β-Man(1 → 4)-GlcNAc; ■, β-Gal-(1 → 4)-β-GlcNAc-(1 → 2)-Man; □, β-GlcNAc-(1 → 6)-Gal; ○, *N*-acetyl-D-glucosamine.

TABLE I

INHIBITION OF DATURA LECTIN-ASIALOFETUIN PRECIPITATION BY LOW MOLECULAR WEIGHT SUGARS

Sugar	[I]mM required for 50% inhibition	Sugar	[I]mM required for 50% inhibition
N-Acetyl-D-Glucosamine	180	β-D-Gal-(1 → 4)-β-D-GlcNAc-(1 → 6)-D-Man	2.0
N,N'-Diacetylchitobiose	2.0	β-D-Gal-(1 → 4)-β-D-GlcNAc (1 → 2)-D-Man	16.0
N,N',N''-Triacetylchitotriose	0.32	*p*-Nitrophenyl (β-D-Gal-(1 → 4)-β-D-GlcNAc-(1 → 2)-β-D-Man*p*	35.0
N,N',N'',N'''-Tetraacetylchitotetraose	0.18	β-D-Gal(1 → 4)-D-Glc	0%, 200 mM
N,N'-Diacetylchitobiitol	0%, 8 mM	β-D-Glc-(1 → 4)-D-Glc	0%, 200 mM
N,N',N''-Triacetylchitotriitol	3.5	β-D-GlcNAc-(1 → 4)-D-Gln	4.6
N,N',N'',N'''-Tetraacetylchitotetritol	0.38	β-D-GlcNAc-(1 → 4)-D-Glc	85
Methyl *N*-acetyl-β-D-glucosaminide	13%, 40 mM	*N*-Trifluoroacetyl, *N'*-acetylchitobiose	2.5
Methyl *N,N'*-diacetyl-β-chitobioside	1.3	*N*-Propionyl, *N'*-acetylchitobiose	2.4
Methyl *N,N',N''*-triacetyl-β-chitotrioside	0.38	*N*-Benzoyl, *N'*-acetylchitobiose	0%, 4 mM
p-Nitrophenyl *N*-acetyl-β-D-glucosaminide	9.0	Methyl *N,N'*-ditrifluoroacetyl-β-chitobioside	4.7
p-Nitrophenyl *N,N'*-diacetyl-β-chitobioside	0.2	Methyl *N,N'*-dipropionyl-β-chitobioside	5.6
p-Nitrophenyl *N,N',N''*-triacetyl-β-chitotrioside	0.24	Chitobiose	0%, 12 mM
p-Nitrophenyl *N*-acetyl-α-D-glucosaminide	11.0	β-D-GlcNAc-(1 → 6)-D-Gal	170
p-Nitrophenyl β-D-galactoside	0%, 8 mM	β-D-Gal-(1 → 6)-D-GlcNAc	0%, 32 mM
β-D-Gal-(1 → 4)-D-GlcNAc	1.2	3,6-di-*O*-(β-D-GlcNAc)-D-Gal	0%, 13 mM
β-D-Gal-(1 → 4)-D-ManNAc	28%, 50 mM	2,6-di-*O*-(β-D-GlcNAc)-D-Man	0%, 3 mM
Me β-D-Gal-(1 → 4)-β-D-GlcN	70.0	2,4 di-*O*-(β-D-GlcNAc)-D-Man	0%, 3 mM
Me β-D-Gal-(1 → 4)-β-D-GlcNProp	6.2	β-D-GlcNAc-(1-*N*)Asn	0%, 12 mM
β-D-Man(1 → 4)-D-GlcNAc	3.0	Methyl 3-*O*-methyl-β-D-GlcNAc*p*	17%, 50 mM
α-D-Man-(1 → 3)-β-D-Man(1 → 4)-D-GlcNAc	2.1	4-*O*-methyl-D-GlcNAc	11%, 50 mM
		Penta-2,4[a]	0.065
		Penta-2,6[a]	0.0025
		Hepta[a]	0.45

[a] Structures shown in Fig. 4.

TABLE II

INHIBITION OF DATURA LECTIN-ASIALOFETUIN PRECIPITATION BY OLIGOSACCHARIDES

Sugar[a]	[I]μM required for 50% inhibition
Penta-2,6	2.5
Nona II	4.6
Aryl Penta-2,6	6.2
Undeca	6.4
Penta-2,4	64
Nona I	120
Hexa-3,6	110
Hepta	450

[a] Structures shown in Fig. 4.

ificity for β-(1 → 4) linked oligomers of *N*-acetyl-D-glucosamine. The values given in Table I for the per-*N*-acetylated chitodextrins are in agreement with this earlier work. *N*-Acetyl-D-glucosamine was a very poor inhibitor, being 90-fold less potent than the corresponding disaccharide. The inhibitory power increased with increasing chain length up to the trisaccharide; *N,N,N''*-triacetylchitotriose was 6 times more inhibitory than *N,N'*-diacetylchitobiose, and *N,N',N'',N'''*-tetraacetylchitotetraose was nearly equivalent to the trisaccharide in inhibitory power, suggesting

that the carbohydrate-binding site can accommodate three sugar residues.

Reduction of the chitin oligomers with sodium borohydride gave the corresponding alditols, containing one less intact sugar ring than the parent compound. This resulted in the reduction of inhibitory potency to that of the next lower homolog. The reduced disaccharide did not inhibit at the highest concentration tested (8 mM), and the reduced trisaccharide was approximately 10-fold poorer than *N,N',N''*-triacetylchitotriose, but only 1.7 times poorer than *N,N'*-diacetylchitobiose. The reduced tetrasaccharide inhibited to almost the same extent as the parent sugar and was of equal potency to *N,N',N''*-triacetylchitotriose. These findings indicate that the open-chain alditol portion of the reduced sugars does not contribute significantly to binding to *Datura* lectin, and also support the hypothesis that *Datura* lectin contains three subsites, and thus optimally binds *N,N',N''*-triacetylchitotriose.

Comparison of the chitin oligomers with their corresponding methyl β-glycosides indicated that the methyl group did not influence binding of the sugar by the protein. In contrast, the presence of the aromatic *p*-nitrophenyl aglycone had a marked effect on the inhibitory activity of the saccharides. *p*-Nitrophenyl β-D-GlcNAc*p* was 20 times more inhibitory than *N*-acetyl-D-glucosamine. *p*-Nitrophenyl *N,N'*-diacetyl-β-chitobioside was 10 times more potent an inhibitor than *N,N'*-diacetylchitobiose, but little enhancement of *p*-nitrophenyl *N,N',N''*-triacetyl-β-chitotrioside relative to the parent sugar was observed. Thus, the *p*-nitrophenyl derivatives of *N,N'*-diacetylchitobiose and *N,N',N''*-triacetylchitotriose were equally good inhibitors, being equivalent to *N,N',N'',N'''*-tetraacetylchitotetraose. The lack of inhibition by *p*-nitrophenyl β-D-galactoside indicates the necessity for an *N*-acetyl-D-glucosamine residue.

The inhibitory power of β-D-Gal-(1 → 4)-D-GlcNAc (*N*-acetyllactosamine) and β-D-Man-(1 → 4)-D-GlcNAc is particularly noteworthy. Both disaccharides differ from *N,N'*-diacetylchitobiose at the C-2 position of the nonreducing sugar, containing an hydroxyl rather than an *N*-acetamido group. The hydroxyl group at C-2 is in the axial position in the mannosyl unit, and in the equatorial position in the galactosyl unit. *N*-Acetyllactosamine further differs from *N,N'*-diacetylchitobiose at C-4 of the nonreducing sugar unit. The configuration of the hydroxyl group at C-4 of this inhibitor is axial, whereas it is in the equatorial position in *N,N'*,-diacetylchitobiose. The inhibitory power of all three disaccharides, however, was very similar. These results argue against the direct involvement of substituents at C-2 and C-4 of the nonreducing sugar in the binding of these disaccharides to *Datura* lectin. Considering the presumed specificity of *Datura* lectin for β-(1 → 4) linked oligomers of *N*-acetyl-D-glucosamine, the noninvolvement of the *N*-acetamido group in the nonreducing sugar was rather surprising. That the remaining *N*-acetamido group is important, however, is apparent from the lack of inhibition by cellobiose and by lactose, even at concentrations of 200 mM.

The trisaccharide α-D-Man-(1 → 3)-β-D-Man-(1 → 4)-D-GlcNAc also exhibited the same inhibitory power as *N,N'*-diacetylchitobiose. Thus, the presence of a substituent at the 3 position of the nonreducing disaccharide unit does not greatly affect binding, and perhaps even enhances it slightly relative to the parent disaccharide, β-D-Man-(1 → 4)-D-GlcNAc. In support of this finding, Pardoe and co-workers (2) have shown that a tetrasaccharide from *Micrococcus lysodeikticus* containing alternating residues of *N*-acetyl-D-glucosamine and *N*-acetylmuramic acid in β-(1 → 4) linkage, is a potent inhibitor of hemagglutination by *Datura* lectin.

The presence of a mannose group at the reducing end of *N*-acetyllactosamine [β-D-Gal-(1 → 4)-β-D-GalNAc-(1 → 6)-D-Man] also did not significantly change its inhibitory power. Inhibitory activity was decreased eightfold, however, if the *N*-acetyllactosamine unit was linked to the C-2 position of mannose, as in β-D-Gal-(1 → 4)-β-D-GlcNAc-(1 → 2)-D-Man.

A series of *N,N'*-diacetylchitobiose derivatives was prepared in which the *N*-acetamido group of the reducing sugar was

modified. Replacement of the *N*-acetamido group with an *N*-trifluoroacetamido or an *N*-propionamido group had no effect on the inhibitory potency of the sugar. In contrast, the *N*-benzamido- derivative did not inhibit at the highest concentration tested (4 mM); limited solubility prevented it from being studied at higher concentrations. The 2-amino-2-deoxy-derivative (β-D-GlcNAc-(1 → 4)-D-GlcN) was a twofold poorer inhibitor than the parent *N,N'*-diacetylchitobiose. However, β-D-GlcNAc-(1 → 4)-D-Glc was not an inhibitor (28% at 50 mM), indicating that the $-NH_2$ group at C-2 of the *reducing* sugar is important in binding. With the exception of the bulky *N*-benzamido derivatives, inhibitory potency is increased when the amino group is present as an amide linkage.

The importance of the acetamido group for binding to *Datura* lectin was further investigated using disaccharide inhibitors in which both *N*-acetyl groups had been modified. Methyl *N,N'*-ditrifluoroacetyl-β-chitobioside and methyl *N,N'*-dipropionyl-β-chitobioside were approximately 3.5 and 4.3 times poorer inhibitors respectively than methyl *N,N'*-diacetyl-β-chitobioside. Chitobiose (β-D-GlcN-(1 → 4)-D-GlcN), with two free $-NH_3^+$ groups, was not an inhibitor at the highest concentration tested (12 mM).

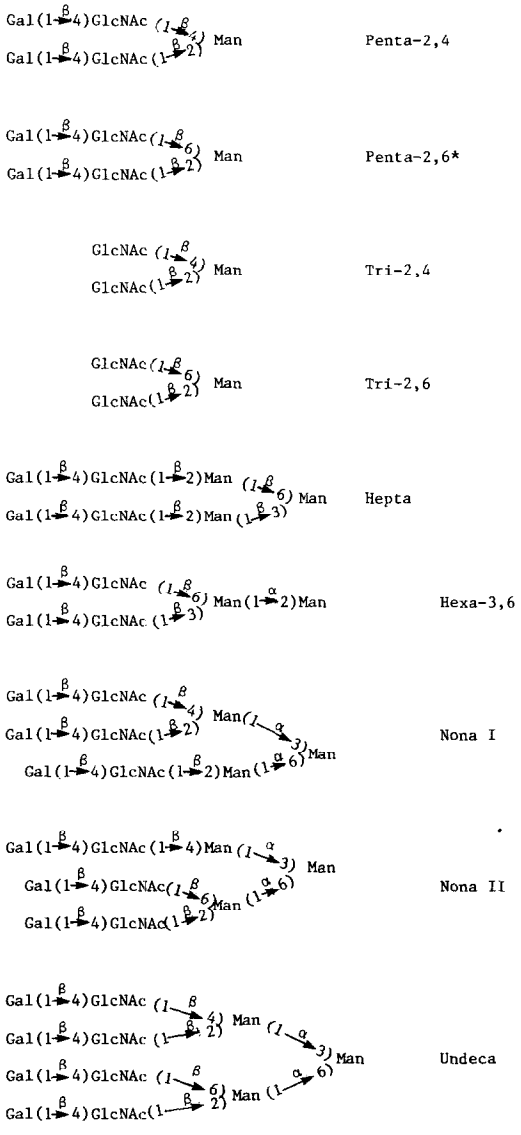
Disaccharides containing β-(1 → 6) linkages were poor inhibitors; β-D-GlcNAc-(1 → 6)-Gal was equivalent to the monosaccharide *N*-acetyl-D-glucosamine in its ability to inhibit the precipitation reaction. The dissacharide D-Gal-(1 → 6)-D-GlcNAc did not inhibit at the highest concentration tested (32 mM). Also noninhibitory were 3,6 di-*O*-(β-D-GlcNAc)-D-Gal; 2,6-di-*O*-(β-D-GlcNAc)-D-Man; and 2,4 di-*O*-(β-D-GlcNAc)-D-Man, although shortage of material prevented these trisaccharides from being tested at very high concentrations. The compound β-D-GlcNAc-(1 → N)Asn did not inhibit precipitation at the highest concentration tested (12 mM).

Methyl 3-*O*-methyl-β-D-GlcNAc*p* and 4-*O*-methyl-D-GlcNAc were equivalent to *N*-acetyl-D-glucosamine in inhibitory potency; at the highest concentration tested (50 mM), 16 and 11% inhibition, respectively, was observed. The lack of inhibition by 4-*O*-methyl-GlcNAc*p* is significant. It indicates the necessity for a sugar substituent at the C-4 position of *N*-acetyl-D-glucosamine.

By far the most potent inhibitors tested were the synthetic oligosaccharides (Fig. 4, Table II), which correspond to portions of the tetraantennary structure of *N*-acetyllactosamine-type glycoproteins. The best inhibitor was Penta-2,6 (2.5 μM), which was 1000 times more potent than *N,N'*-diacetylchitobiose, and almost 10^5 times more potent than *N*-acetyl-D-glucosamine. Nona II, a triantennary oligosaccharide not found in nature, and Undeca, a tetraantennary oligosaccharide, were nearly equivalent to Penta-2,6 in inhibitory potency (4.6 and 6.4 μM, respectively). It is noteworthy that the Penta-2,6 unit is a structural component of both these oligosaccharides (Nona II and Undeca). Penta-2,4, Nona I (which contains a Penta-2,4 group moiety), and Hexa-3,6 were of similar inhibitory potency, and approximately 25–50 times less potent than Penta-2,6. The heptasaccharide was 180 times less potent as an inhibitor than Penta-2,6, and only 4.4 times more potent than *N,N'*-diacetylchitobiose.

DISCUSSION

We rationalize the results of our hapten inhibition studies between the *D. stramonium* lectin and naturally occurring and chemically modified mono- and oligosaccharides as follows: the binding site of *D. stramonium* is composed of three subsites, designated A, B, and C (Fig. 5), a situation reminiscent of wheat germ agglutimin (28) and the potato lectin (29, 30). A disaccharide can potentially bind to the lectin in two ways, occupying subsites A and B, or subsites B and C. We propose that the minimal requirements for binding include an equatorial acetamido group at C-2 of the sugar occupying subsite B; an amino group at C-2 of the sugar greatly diminishes its binding affinity. Additionally, the sugar occupying subsite B must be present in a β-(1 → 4) linkage. There is considerable freedom in the allowed configuration of the sugar occupying subsite A. An *N*-acet-



*Aryl Penta-2,6 has an α-linked p-$C_6H_4NHCOCF_3$ aglycone

FIG. 4. Structures of synthetic oligosaccharides assayed for their ability to inhibit the precipitation reaction between *Datura* lectin and asialofetuin.

amido group is not necessary for binding to this subsite. Thus, disaccharides containing a single β-(1 → 4)-linked GlcNAc residue in the reducing position, such as β-D-Gal, Man-(1 → 4)-D-GlcNAc, would bind to subsites A and B, respectively. The proposed interaction of several sugars with the lectin's subsites is illustrated in Fig. 5.

Although consistent with the hapten inhibition results, this model is obviously limited by the availability of structurally relevant compounds. To further define the important structural features involved in binding site interactions, further oligosaccharides must be tested. For example, all of the sugars tested contained an equatorial hydroxymethyl group at C-6 and an equatorial hydroxyl group at C-4. Modification of these groups would be expected to yield important information about the specificity requirements of the *D. stramonium* binding site.

The nature of the interaction between *Datura* lectin and the aromatic aglycone portion of a given sugar also remains unclear. Hapten inhibition studies indicated that the *p*-nitrophenyl glycosides of *N*-acetyl-D-glucosamine and its β-(1 → 4) linked oligomers were potent inhibitors. The in-

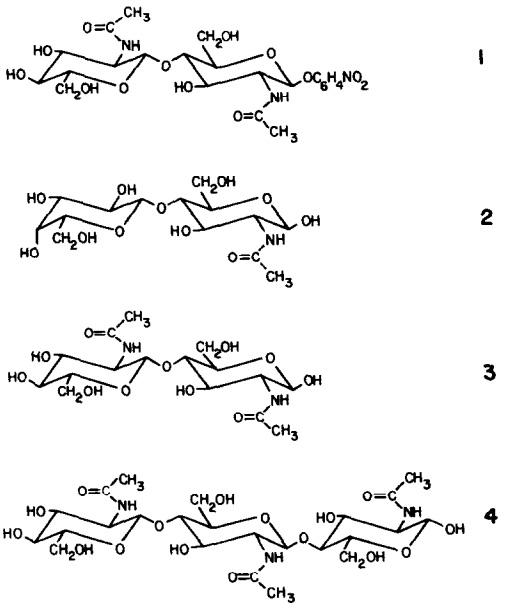


FIG. 5. Proposed three-subsite model of the *Datura* lectin-binding site indicating the subsites occupied by the indicated sugars: 1, *p*-nitrophenyl *N,N'*-diacetyl-β-chitobioside; 2, *N*-acetyllactosamine; 3, *N,N'*-diacetylchitobiose; 4, *N,N'N"*-triacetylchitotriose.

hibition was specific, inasmuch as *p*-nitrophenyl β-D-galactoside was a noninhibitor. In both uv difference spectroscopy and fluorescence spectroscopy, however (Crowley and Goldstein, unpublished results), no specific perturbations of the aglycone could be detected. In addition, no positive peak in the uv difference spectrum at 295 nm was observed with the *p*-nitrophenyl glycoside, as is seen when free *N,N'*-diacetylchitobiose interacts with *Datura* lectin. We propose that a hydrophobic interaction occurs between the *p*-nitrophenyl aglycone of *N*-acetyl-D-glucosamine and *N,N'*-diacetylchitobiose, both of which exhibit enhanced binding compared to their parent sugars, and a region on the *Datura* lectin between subsites B and C. According to this scheme (Fig. 5) the monomer would occupy subsite B and the dimer subsites A and B, respectively. This would also account for the fact that both *p*-nitrophenyl *N,N',N''*-triacetyl-β-chitotrioside and *p*-nitrophenyl β-D-Gal-(1 → 4)-β-D-GlcNAc-(1 → 2)-β-D-Man*p*, which would occupy subsites A, B and C, showed no enhanced binding.

Additional information about the binding-site specificity of *Datura* lectin was obtained using a series of branched-chain oligosaccharides as inhibitors. The structures of these oligosaccharides (see Fig. 4) correspond to portions of the tetraantennary structure of *N*-acetyllactosamine-type glycoproteins. The most striking feature of these branched-chain oligosaccharides is their remarkably high inhibitory potency relative to the component di and tri units (β-D-Gal-(1 → 4)-D-GlcNAc, and β-D-Gal-(1 → 4)-β-D-GlcNAc-(1 → 2)-D-Man; see Tables I, II). Penta-2,6 in particular, as well as Nona II and Undeca, which contain a penta 2,6-moiety, were 2–3 orders of magnitude more potent inhibitors than *N,N'*-diacetylchitobiose. Compounds containing a penta-2,4 or hexa-3,6 moiety were 25–50 times less potent. One explanation for these results is that a biantennary saccharide, such as Penta-2,6, could occupy both binding sites of *Datura* lectin simultaneously. Because the free energy of binding (ΔG) values are additive, and exponentially related to the K_d (dissociation constant) values, it follows that the simultaneous occupation of both binding sites would result in a large increase in affinity for that oligosaccharide. This hypothesis has also been suggested by Kronis and Carver (31) to account for the difference in binding affinity of WGA for simple sugars compared to glycoconjugates present on cell surfaces. For example, the K_d value for the binding of *N,N',N'',N'''*-tetraacetylchitotetritol by *Datura* lectin, as measured by equilibrium dialysis, is 1.2×10^{-4} M ($K_a = 8.7 \times 10^3$ M^{-1}). From the hapten inhibition studies reported in this work, it was shown that *N*-acetyllactosamine is about a threefold poorer inhibitor than *N,N',N'',N'''*-tetraacetylchitotetritol. Therefore, the K_d for *N*-acetyllactosamine can be roughly approximated to be 4×10^{-4} M, which corresponds to a ΔG value of −4.7 kcal/mol. The ΔG for binding two *N*-acetyllactosaminyl units would thus be −9.3 kcal/mol, which corresponds to a K_d value of 1.6×10^{-7} M. (If the K_d for a single *N*-acetyllactosaminyl unit was 1×10^{-3} M, the K_d for two such units would be 1×10^{-6} M). Thus, the simultaneous occupation of both lectin-binding sites can account for an increased association constant of 2–3 orders of magnitude. Indeed, penta-2,6 is approximately 500 times more potent an inhibitor than *N*-acetyllactosamine. Furthermore, preliminary results obtained by uv difference spectroscopy indicate that the affinity constant for penta-2,6 is $1\text{–}2 \times 10^{-6}$ M.

This study confirms our previous report (8) that the *D. stramonium* lectin is unique in binding *N*-acetyllactosamine with high affinity. Only one other agglutinin, the D-galactose/*N* - acetyl - D - galactosamine - binding lectin present in *Erythrina crystagalli* seeds (32), also interacts strongly with this disaccharide unit. It is indeed interesting that the *Datura* lectin appears to have the same specificity for the penta-2,6 oligosaccharide as leucoagglutinin (L-PHA), the L_4 isolectin of the red kidney bean, *Phaseolus vulgaris* (33, 34). Since *N*-acetyllactosamine is a structural component of many glycoproteins, reactivity with *Datura* lectin may have important implications regarding the use of *Datura* lectin as a biochemical tool.

This study also reports the high-affinity interaction of *Datura* lectin with several complex oligosaccharides. Preliminary results suggest that these biantennary oligosaccharides bind to both lectin-binding sites simultaneously. This hypothesis is currently being tested in our laboratory.

Note added in proof: Immobilized *Datura stramonium* lectin has been used to bind poly-*N*-acetyllactosamine-type glycopeptides [Cummings, R. D. and Kornfeld, S. (1984) *J. Biol. Chem.* **259,** 6253-6260].

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