Carbohydrate Binding Specificity of Four *N*-Acetyl-D-galactosamine-"Specific" Lectins: *Helix pomatia* A Hemagglutinin, Soy Bean Agglutinin, Lima Bean Lectin, and *Dolichos biflorus* Lectin†

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ABSTRACT: The detailed carbohydrate binding specificity of four *N*-acetyl-D-galactosamine-binding proteins, *Helix pomatia* A hemagglutinin (HP), soy bean agglutinin (SBA), *Dolichos biflorus* (DB), and lima bean lectin (LBL), was compared using the same saccharides for precipitation-inhibition and the same model macromolecules for direct precipitation. The analyses allowed a determination of the probable size of their combining sites, a definition of their specificity ranges, and the identification of several groups (hydroxyl and acetamido) in *N*-acetyl-D-galactosamine probably involved in hydrogen-bond formation with amino acid residues in the binding sites of these proteins. The combining site of all four carbohydrate binding proteins corresponds to the size of a monosaccharide. The following specificity ranges were found: HP (α-D-GalNAc*p* > α-D-GlcNAc*p* ≃ β-D-GalNAc*p* > β-D-GlcNAc*p* > α-D-Gal*p*); SBA (α,β-D-GalNAc*p* > α,β-D-Gal*p*); DB (α-D-GalNAc*p* > β-D-GalNAc*p*); LBL (α-D-GalNAc*p* > β-D-GalNAc*p*). Two contact groups in the *N*-acetyl-D-galactosamine molecule, probably involved in hydrogen-bond formation with the HP-binding site, were identified: the carbonyl oxygen on the C-2 acetamido group and the O atom on the C-4 hydroxyl group. For SBA three contact groups were identified: the carbonyl oxygen on the C-2 acetamido group and the H atoms on the C-4 and C-6 hydroxyl groups. For DB and LBL only the C-2 acetamido group in D-GalNAc*p* has been identified as being in contact with the binding site of these lectins.

In this paper we report a *comparative* study on the carbohydrate-binding specificity of four *N*-acetyl-D-galactosamine-binding lectins. The lectins are *Helix pomatia* A hemagglutinin (HP),[1] soy bean agglutinin (SBA), *Dolichos biflorus* lectin (DB), and lima bean lectin (LBL). Earlier studies by us (Hammarström and Kabat, 1969, 1971; Hammarström et al., 1972; Etzler and Kabat, 1970; Galbraith and Goldstein, 1972) and by Lis et al. (1970) and Pereira et al. (1974) have outlined the general specificity of these lectins. In this study the analysis has been carried further. The four lectins have been compared by using the same saccharides for precipitation-inhibition and the same model macromolecules for direct precipitation. The data allow a definition of the specificity range for HP, SBA, LBL, and DB and a tentative identification of the groups (hydroxyl, acetamido) within the *N*-acetyl-D-galactosamine molecule which probably are involved in hydrogen-bond formation with amino acid residues in the binding sites of these lectins.

Experimental Section

Materials

Lectins. Purified *Helix pomatia* A hemagglutinin (HP), *Dolichos biflorus* lectin (DB), lima bean lectin (LBL), and soy bean agglutinin (SBA) were prepared by specific absorption to columns of insolubilized hog blood group A + H substance (polyleucyl-hog A + H substance) followed by specific elution with *N*-acetyl-D-galactosamine. The detailed procedures for the purification of HP, DB, and LBL have been described previously (Hammarström and Kabat, 1969; Etzler and Kabat, 1970; Galbraith and Goldstein, 1972). All four lectins were homogeneous on gel filtration and completely precipitated by human blood group A substance (or hog A + H substance). Their chemical and physicochemical properties have been described in previous communications (Hammarström and Kabat, 1969, 1971; Hammarström et al., 1972; Etzler and Kabat, 1970; Carter and Etzler, 1975 a-c; Galbraith and Goldstein, 1972; Lotan et al., 1974). Purified *Ricinus communis* lectin (RCA_I) was prepared as previously described (Nicolson et al., 1974).

Glycoproteins and Polysaccharides. Purified hog gastric mucin with A + H blood group activities, human blood group A substance from ovarian cysts and polyleucyl hog A + H substance were available from previous studies (Hammarström and Kabat, 1969; Galbraith and Goldstein, 1972). The galactomannan of *Cyamopsis tetragonolobus* (guaran) was obtained from the Meer Corp. (New York, N.Y.) and was further purified as earlier described (Hayes and Goldstein, 1974). Purified *Larix occidentalis* arabinogalactan B (molecular weight ~16 000) was a gift from Dr. L. Kenne of the University of Stockholm. Carcinoembryonic antigen (CEA) was purified as described by Hammarström et al. (1976). The

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[1] Abbreviations used: HP, *Helix pomatia* A hemagglutinin; SBA, soy bean agglutinin; DB, *Dolichos biflorus* lectin; LBL, lima bean lectin; CEA, carcinoembryonic antigen; CEA-SI, product obtained by Smith degradation from CEA; Me α- and β-D-GalNAc*p*, methyl 2-acetamido-2-deoxy-α- and β-D-galactopyranosides, respectively; Me α- and β-D-GlcNAc*p*, methyl 2-acetamido-2-deoxy-α- and β-D-glucopyranoside, respectively.

chemical and immunochemical properties of purified CEA and the product obtained by Smith degradation (CEA-SI) have been described (Hammarström et al., 1975, 1976). Some of the carbohydrate–bovine serum albumin conjugates were available from earlier studies (Iyer and Goldstein, 1973; Buss and Goldstein, 1968). The *p*-azophenyl 2-acetamido-2-deoxy-α- and β-D-galactopyranoside–BSA conjugates were prepared similarly. All conjugates contained 15 to 22 sugar units/albumin molecule.

Sugars. Methyl 2-acetamido-2-deoxy-α- and β-D-galactopyranosides (Me α- and β-D-GalNAc*p*), methyl 2-acetamido-2-deoxy-α- and β-D-glucopyranoside (Me α- and β-D-GlcNAc*p*), and methyl 2-acetamido-2-deoxy-β-D-galactofuranoside were prepared by incubating the free sugars with Dowex-50 (H^+) and methanol in the cold. The anomeric glycosides were separated according to Neuberger and Wilson (1971). Methyl 2-acetamido-2-deoxy-β-D-galactofuranoside (not reported by Neuberger and Wilson, 1971) was recovered following elution of the pyranosides. Its structure was confirmed by mass spectrometry (Jacquinet and Sinay, 1974). Methyl 2-deoxy-2-(*p*-nitrobenzamido)-α-D-galactopyranoside, methyl 2-deoxy-2-(bromoacetamido)-α-D-galactopyranoside and -glucopyranoside, and methyl 2-deoxy-2-amino-α-D-galactopyranoside were prepared by Dr. D. H. Buss, University of Michigan. 4-Deoxy-4-fluoro-D-galactose and 6-deoxy-6-fluoro-D-galactose were kindly donated by Dr. J. Westwood, Chester Beatty Institute, London; 1,5-anhydro-D-galactitol was a gift of Dr. W. Jakinovich, University of Michigan; *N*-acetyllactosamine and methyl β-D-mannopyranoside were gifts from Dr. G. W. Jourdian, University of Michigan, and Dr. P. Garegg, University of Stockholm, respectively. All other sugars used were commercially available and most of them were purchased from Pfanstiehl Laboratories.

Methods

Precipitation Analysis. Quantitative precipitin analyses were performed by a microprecipitin technique (Kabat, 1961), employing a final volume of 200 μL. The tubes were incubated at 4 °C (except for LBL which was incubated at 37°C) for 1 week. Nitrogen in the washed precipitates was determined by a ninhydrin procedure (Schiffman et al., 1964). Lectin protein, 30–50 μg, was used per tube.

Inhibition of quantitative precipitation was performed by the addition of increasing amounts of inhibitor (prior to addition of glycoprotein) to the "equivalence mixture" of lectin and glycoprotein in a total volume of 200 μL. The tubes were processed and analysed as above. The following equivalence mixtures were utilized: 27 μg of HP and 12 μg of galactomannan; 39.5 μg of SBA and 10 μg of galactomannan; 32.5 μg of DB and 35 μg of hog A + H substance; 50 μg of LBL-III and 14 μg of human blood group A substance.

Results

Direct Precipitation. The results of a large number of precipitin analyses with HP, SBA, DB, LBL-II, and LBL III and biopolymers or carbohydrate–bovine serum albumin conjugates are summarized in Figure 1. The precipitin curves generated by the addition of hog A + H substance are shown for each lectin as a reference. As can be seen all four lectins precipitated with hog A + H substance (i.e., with a macromolecule containing multiple, nonreducing α-linked D-GalNAc end groups). Previous studies have demonstrated that terminal nonreducing α-D-GalNAc units in the A determinant are exclusively or predominantly responsible for the precipitation reactions between HP or DB and human blood group

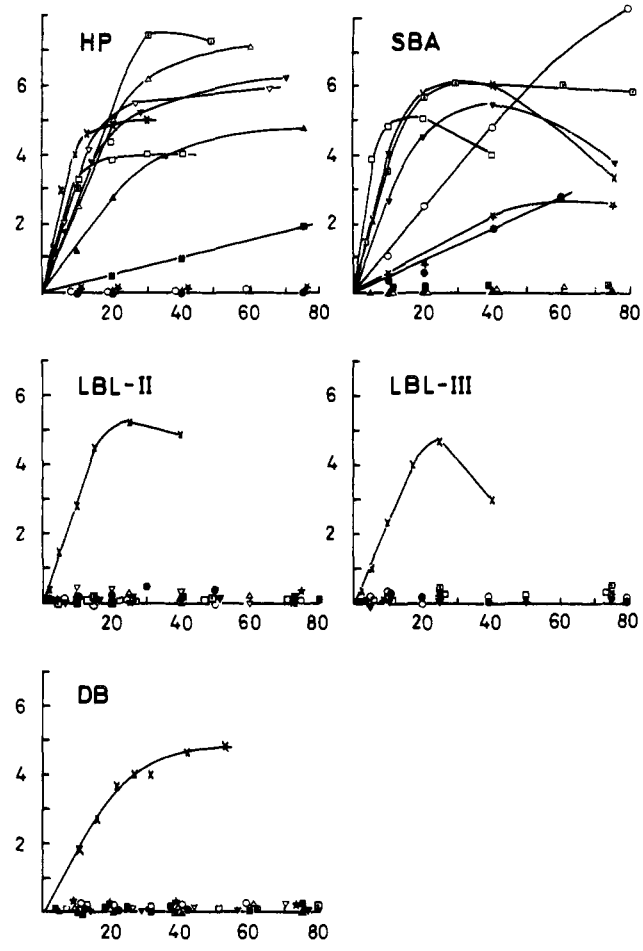

FIGURE 1: Precipitation of biopolymers and carbohydrate–bovine serum albumin conjugates by *Helix pomatia* A hemagglutinin (HP), soy bean agglutinin (SBA), *Dolichos biflorus* lectin (DB), and lima bean lectin II and III (LBL II and III). Four micrograms N of HP, 5.9 μg N of SBA, 4.9 μg N of DB, and 4.9 and 4.8 μg N of LBL II and III, respectively, were added per tube; the total volume was 200 μL. Abscissa: μg N in precipitate. Ordinate: μg biopolymer or carbohydrate–bovine serum albumin conjugate added. (X) Hog A + H substance; (□) guaran; (⊡) *p*-azophenyl α-D-GalNAc*p*-BSA; (▽) methyl 2-deoxy-2-(*p*-azobenzamido)-α-D-Gal*p*-BSA; (▼) *p*-azophenyl-β-D-GalNAc*p*-BSA; (▲) *p*-azophenyl β-D-GlcNAc*p*-BSA; (■), methyl 2-deoxy-2-(*p*-azobenzamido)-α-D-Glc*p*-BSA; (○) *p*-azophenyl β-D-Gal*p*-BSA; (●) CEA; (△) CEA SI; (★) *p*-azophenyl α-D-Gal*p*-BSA.

A substance, respectively (Hammarström and Kabat, 1969; Etzler and Kabat, 1970). Interestingly, of the four lectins, only HP and SBA precipitated the α- and β-D-GalNAc-BSA conjugates (*p*-azophenyl 2-acetamido-2-deoxy-α- and β-D-galactopyranoside–BSA). Two macromolecules with terminal nonreducing β-D-GlcNAc end groups also were investigated (*p*-azophenyl 2-acetamido-2-deoxy-β-D-glucopyranoside–BSA and CEA-SI). HP was the only lectin which precipitated with these two substances. Two macromolecules with terminal, nonreducing α-D-galactopyranosyl end groups (guaran and *p*-azophenyl α-D-galactopyranoside–BSA) were tested. The galactomannan (guaran) was precipitated by HP and SBA and the α-D-Gal–BSA conjugate only by SBA. Four macromolecules with terminal, nonreducing β-D-galactopyranosyl end groups (*p*-azophenyl β-D-galactopyranoside–BSA, *p*-azophenyl β-lactoside–BSA, CEA, and an arabinogalactan from *L. occidentalis*) were also investigated. HP, DB, and LBL did not precipitate with any of the four substances, whereas SBA precipitated with β-D-Gal–BSA and CEA, but not with the other two macromolecules. β-Lactoside–BSA, the arabino-

TABLE I: Inhibition of *Helix pomatia* A Hemagglutinin, Soy Bean Agglutinin, *Dolichos biflorus* Lectin, and Lima Bean Lectin Precipitation by Saccharides.

Saccharide	Inhibitor (nmol/200 μL) for 50% inhibition			
	HP	SBA	DB	LBL-III
Me α-D-GalNAc*p*	2.2	4.5	200	2 600
Me β-D-GalNAc*p*	24	2.5	1 100	17 000
D-GalNAc	3.4	2.5	650	8 000
Me β-D-GalNAc*f* (furanoside)	>100	>500	>20 000	>60 000
Me (*N*-bromoacetyl)-α-D-GalN*p*	1.9	2.0	260	2 500
Me (*N*-*p*-nitrobenzamido)-α-D-GalN*p*	20	ND	>1 000	510
Phenyl α-D-GalNAc*p*	3.5	ND	300	2 100
o + *p*-nitrophenyl α-D-GalNAc*p*	6.0	ND	300	3 000
p-Nitrophenyl β-D-GalNAc*p*	32	0.9	>600	>8 700[a]
Me α-D-GalN*p*	7 700	ND	>20 000	>130 000[a]
D-GalN	>18 600	600	>4 250	[b]
N-Acetyl-D-galactitol	>10 000	>5 000	>5 000	ND
Me α-D-GlcNAc*p*	22	>1 350	>30 000	>230 000
Me β-D-GlcNAc*p*	120	>1 350	>15 000	>290 000
D-GlcNAc	23	>5 000	>10 000	158 000
D-GlcN	>35 000	ND	ND	[b]
Me α-D-Gal*p*	1 200	110	30 000	34 400
Me β-D-Gal*p*	5 000	150	~75 000	200 000
D-Gal	3 800	165	~75 000	38 000
2-Deoxy-D-Gal	5 500	1 800	>100 000	74 000
4-Deoxy-D-Gal	>10 100	ND	>11 000	ND
4-Deoxy-4-fluoro-D-Gal	10 500	>1 800	>7 000	ND
D-Fuc	8 500	1 800	27 000	45 000
L-Ara	5 000	4 000	ND	ND
6-Deoxy-6-fluoro-D-Gal	>8 500[a]	1 800	ND	ND
Melibiose	710	70	22 000	28 400
Raffinose	200	200	ND	>100 000
Stachyose	750	180	ND	ND
Lactose	10 000	230	25 000	51 000
N-Acetyllactosamine	>1 000	150	ND	ND
Me α-D-Man*p*	20 000	>3 000	>80 000	170 000
Me β-D-Man*p*	>15 000	>3 000	ND	>290 000
Me α-D-Glc*p*	6 000	>3 000	>100 000	>300 000
Me β-D-Glc*p*	12 000	>3 000	ND	>310 000
Me α-D-Fuc*p*	>20 000	>3 000	ND	ND
D-ManNAc	500	>5 000	>7 000	>85 000
1,5-Anhydro-D-galactitol	1 600	ND	ND	42 400
D-Allose	>15 000	>15 000	48 000	ND

[a] Inhibitions (20–30%) were obtained at these concentrations. [b] Decomposition of D-GalN and D-GlcN occurred.

galactan, and CEA were all strongly precipitated by *Ricinus communis* lectin (RCA_I) confirming the presence of terminal, nonreducing β-D-galactopyranosyl end groups in these substances.

One BSA conjugate, in which methyl 2-deoxy-2-amino-α-D-galactopyranoside was conjugated to BSA via an *N*-benzamidoazo linkage (methyl 2-deoxy-2-(*p*-azobenzamido)-α-D-galactopyranoside–BSA) was tested against all lectins except SBA. Only HP was found to precipitate with this conjugate. A similar conjugate in which methyl 2-amino-2-deoxy-α-D-glucopyranoside was conjugated to BSA precipitated only weakly with HP and not at all with the other three lectins.

Inhibition of Precipitation. A large number of monosaccharides, methylglycosides, and oligosaccharides, either structurally related to D-GalNAc or occurring as constituents of mammalian glycoproteins or glycolipids, were investigated as inhibitors of precipitation between the four lectins and their corresponding "antigens". In order to obtain high sensitivity, precipitation systems were set up in which the interaction between the lectin and the macromolecule was weak. For HP and SBA two very sensitive systems were obtained by reacting the lectins with the galactomannan, guaran. Two and five nanomoles/200 μL of Me α-D-GalNAc*p* were needed for 50% inhibition of HP- and SBA-galactomannan precipitation, respectively. The corresponding values for Me α-D-GalNAc*p* in the HP- or SBA-blood group A substance systems were 500–1000 nmol/200 μL (Hammarström and Kabat, 1971; S. Hammarström, unpublished results). Since DB only precipitated macromolecules with terminal, nonreducing α-linked D-GalNAc end groups (blood group A substance, etc.) (Figure 1) (Etzler and Kabat, 1970), no precipitating system with increased sensitivity was available. Consequently the inhibition studies were performed in the DB–blood group A substance system. Similarly, LBL was reacted against blood group A substance for the same reasons. As can be seen from Table I, the latter two systems were relatively insensitive, 200 and 2600 nmol of Me α-D-GalNAc*p* were required for 50% inhibition of DB and LBL–III precipitation, respectively. The inhibition data in Table I are presented in terms of the amounts of sugar required for 50% inhibition. The values were obtained from *complete* inhibition curves.

With but two exceptions, namely, methyl 2-deoxy-2-(*p*-nitrobenzamido)-α-D-galactopyranoside in the LBL system and *p*-nitrophenyl β-D-GalNAc*p* in the SBA system (see below), Me α-D-GalNAc*p* was the best inhibitor tested in all four lectin systems. The fact that D-galactosamine itself is a poor inhibitor of the lectin systems suggests that an $-NH_3^+$ group at the C-2 position is not tolerated in the lectins' combining sites.

An interesting difference between the carbohydrate binding properties of HP, DB, and LBL, on the one hand, and SBA, on the other, was observed upon comparing the inhibiting capacity of 2-deoxy-D-galactose with D-galactose. The two sugars were approximately equally effective as inhibitors in the HP-, DB-, and LBL-precipitating systems, whereas, in the SBA system, D-galactose was about ten times more active than 2-deoxy-D-galactose. This indicates that the equatorially oriented hydroxyl on C-2 in D-galactose contributes positively to the binding between the SBA site and the sugar and may explain the relatively strong reactivity of SBA with D-galactose. The data also indicate that D-GalNAc must be in the pyranose ring form ($^4C_1$) in order to interact with the binding site of HP, SBA, DB, or LBL; Me β-D-GalNAc*f* (furanoside) and 2-acetamido-2-deoxy-D-galactitol were noninhibitors even at high concentrations. With but one exception, none of the oligosaccharides investigated in this study was a better inhibitor than the corresponding methyl glycoside of the nonreducing glycosyl group of the oligosaccharide. The exception was raffinose which was about six times more active than Me α-D-Gal*p* in the HP–galactomannan precipitating system. The methyl α-D-glycosides (or nitrophenyl α-D-glycosides) were better inhibitors than the corresponding β-glycosides in the HP-, DB-, and LBL-precipitating systems, whereas there were no significant differences in inhibitory capacity between α- or β-linked methyl glycosides in the SBA system. Phenyl or *p*-nitrophenyl α- and β-glycosides of D-GalNAc were no better inhibitors than the corresponding methyl α- or β-glycosides in the HP-, DB-, and LBL-precipitating systems. However, in the SBA system, *p*-nitrophenyl β-D-GalNAc*p* was found to be three times more potent as an inhibitor than Me β-D-GalNAc*p*. A similar finding was made by Pereira et al. (1974), who demonstrated that phenyl α-D-GalNAc*p* was approximately two times more active than Me α-D-GalNAc*p*. 1,5-Anhydro-D-galactitol was approximately as inhibitory as Me α-D-Gal*p* in the HP- and LBL-precipitating systems, indicating that there is no positive contribution by the axially oriented oxygen at C-1, to the interaction between sugar and HP or LBL binding sites. The four lectins behaved differently with respect to the ease by which D-GlcNAc and its methyl glycosides inhibited their precipitation. Of the four lectins, only HP precipitation was relatively easy to inhibit by D-GlcNAc, Me α-D-GlcNAc*p*, and Me β-D-GlcNAc*p* (5–20 times higher concentrations than for D-GalNAc and its methyl glycosides were needed for 50% inhibition). SBA, LBL, and DB precipitations, on the other hand, were not inhibited at all by D-GlcNAc or its methyl glycosides, even at very high concentrations. These findings indicate that an axially oriented hydroxyl group at the C-4 position of D-GalNAc is absolutely essential for binding to SBA, LBL, and DB. For HP, axial orientation of the C-4 hydroxyl is preferred but not absolutely essential.

Additional information on the contribution to binding of the axially oriented hydroxyl group on C-4 was obtained by comparing the inhibition potency of 4-deoxy-D-galactose and 4-deoxy-4-fluoro-D-galactose with that of D-galactose. As can be seen, HP precipitation was inhibited by the fluoro sugar at about the same concentration as with D-galactose, whereas the 4-deoxy sugar was active; this suggests that the *oxygen* atom of the C-4 hydroxyl group probably forms a hydrogen bond with the combining site of HP (for details on this type of analysis, see Goldstein et al., 1974). SBA precipitation was inhibited very poorly by 4-deoxy-4-fluoro-D-galactose (at least ten times higher a concentration was required than for D-galactose), indicating that the *hydrogen* atom of the C-4 hydroxyl group probably forms a hydrogen bond with the protein. For DB and LBL this information could not be obtained due to the high amount of sugars needed for inhibition. The contribution to binding of the C-6 hydroxymethyl group in D-galactose was determined by comparing the inhibition power of D-fucose (6-deoxy-D-galactose), L-arabinose, and 6-deoxy-6-fluoro-D-galactose with D-galactose. Data were obtained only for HP and SBA. Since there was no significant difference in inhibition power between these four sugars in the HP system, it was concluded that the C-6 hydroxymethyl group makes little, if any, contribution to binding. On the other hand, the data indicate that the C-6 hydroxyl group makes a positive contribution to binding in the SBA system; and furthermore, it is probably the hydrogen atom which participates in hydrogen bond formation with the site. Methyl α- and β-glycosides of D-mannose and D-glucose or Me α-L-Fuc*p* did not inhibit or were extremely poor inhibitors in all four lectin systems. Finally, two potential affinity labeling compounds, methyl 2-deoxy-2-bromoacetamido-α-D-galactopyranoside and methyl 2-deoxy-2-(*p*-nitrobenzamido)-α-D-galactopyranoside (the latter reactive after conversion to the corresponding diazo or arylazido derivative), proved to be good to excellent inhibitors in the lectin precipitation systems investigated.

Discussion

The data on the specificity of the carbohydrate binding sites of the four lectins, obtained in this study, and those referred to in the introductory section will be discussed under three headings: size of carbohydrate binding site, contact groups, and specificity range.

Size of Carbohydrate Binding Site. Available data suggest that the binding site of all four lectins is relatively restricted, probably corresponding to the size of a single monosaccharide unit. Of all oligosaccharides containing D-GalNAc, D-GlcNAc, and D-galactose as the terminal nonreducing sugar (including a large number of blood-group-active oligosaccharides), only two were found to be significantly better inhibitors than the methyl α- or β-glycoside of the sugar corresponding to the terminal nonreducing sugar of the oligosaccharide (variations in the relative inhibition power between saccharide of a factor of 2–3 are within the experimental error of the assay). The two exceptions were both found in the HP system. A *S. typhimurium* lipopolysaccharide core hexa- or heptasaccharide, containing two terminal, nonreducing α-linked D-galactose residues (Hammarström et al., 1972), and raffinose [α-D-Gal*p*-(1→6)α-D-Glc*p*-(1→2)-D-Fru*f*] (Table I) were ten and six times more inhibitory than Me α-D-Gal*p*, respectively. The significance of these findings is unclear. However, it should be pointed out that these two oligosaccharides are still relatively poor inhibitors compared with Me α-D-GalNAc*p*.

Contact Groups. The inhibition data clearly demonstrate that *N*-acetyl-D-galactosamine is the monosaccharide which is most complementary to the binding site of all four lectins. The data also show that the sugar must be in the pyranose form in order to interact with the binding sites (Table I). It is

probable that the sugar exists in the $^4C_1$ chair conformation when bound to the combining site of the lectins since this is the normal conformation for Me α-D-GalNAc*p* and Me β-D-GalNAc*p*, when in solution. SBA interacts most strongly with *N*-acetyl-D-galactosamine, $K_0 = 3 \times 10^4$ M^{-1}, followed by HP, $K_0 = 5 \times 10^3$ M^{-1}, and LBL $K_0 = 1 \times 10^3$ M^{-1}. All values were measured at 20 °C and neutral pH (Lotan et al., 1974; Hammarström and Kabat, 1971; Bessler and Goldstein, 1974). The intrinsic association constant for the interaction between DB and D-GalNAc has not yet been determined precisely. However, preliminary experiments indicate that the value is relatively low and probably close to that found for LBL.

The analysis of which groups in the *N*-acetyl-D-galactosamine molecule are in contact with the carbohydrate binding sites of the lectins is based on the following assumptions: (1) hydrogen bonds are the major forces responsible for binding of neutral sugars to the sites; (2) by comparing the inhibition power of a monodeoxy sugar with that of the parent sugar (for example, 4-deoxy-D-galactose and D-galactose), it can be established whether or not a particular hydroxyl (or acetamido) group contributes to the interaction; (3) a fluorine atom, when substituted for a hydroxyl group in a sugar may participate in hydrogen-bond formation comparable in function to the oxygen atom of a hydroxyl group; (4) a sugar which lacks a particular group is accommodated in the site in the same way as the parent sugar. The general validity of the first three assumptions has been documented in studies of the lysozyme–hexa-*N*-acetylchitohexaose interaction (Phillips, 1967) and in studies of the interaction between concanavalin A and model sugars (Poretz and Goldstein, 1970; Goldstein et al., 1974).

HP. On the basis of these and previous data, we suggest the following two contact groups: the carbonyl oxygen on the equatorially oriented acetamido group at C-2 and the O atom of the axially disposed C-4 hydroxyl. The assignment of the carbonyl oxygen as a contact group is based on the following findings: Me 2-*O*-acetyl-α-D-Gal*p* and Me α-D-GalNAc*p* are both excellent inhibitors of HP precipitation (Hammarström, 1972). D-Galactose and 2-deoxy-D-galactose, on the other hand, are poor inhibitors. Furthermore there is no difference in the inhibition potency of the latter two compounds, indicating that the hydroxyl group on C-2 in D-galactose does not contribute to binding. This implies that the carbonyl oxygen is responsible for the increased binding affinity of Me 2-*O*-acetyl-α-D-Gal*p* as compared with D-Gal. Since the carbonyl oxygen occupies a sterically similar position in 2-*O*-acetyl-D-Gal and D-GalNAc, it may be assumed that this atom also is in contact with the binding site in the latter sugar.

The C-6 hydroxyl group and the α-anomeric oxygen at C-1 do not appear to be involved in the binding mechanism. The preference of HP for α anomers as compared with β anomers appears to depend on the destabilizing effect of the latter on the interaction. No information is available on the contribution to binding of the equatorially oriented hydroxyl at C-3.

SBA. Three contact groups were tentatively identified: the *N*-acetamido group at C-2 (probably the carbonyl oxygen) and the hydrogen atoms of the C-4 and C-6 hydroxyl groups. As for HP, it was not possible to evaluate the contribution to binding of the hydroxyl group at C-3. Furthermore, no information is available on the contribution to binding of the anomeric oxygen at C-1.

D-Galactose is a relatively good inhibitor in the SBA system, whereas this sugar is a poor inhibitor in the other lectin systems. By comparing the inhibition power of D-galactose with 2-deoxy-D-galactose (Table I), it can be seen that the C-2 hydroxyl in D-galactose makes a positive contribution to binding to the SBA site but not to the HP or LBL sites. The relatively strong interaction of D-galactose with SBA may possibly be explained by this additional interaction.

Finally, there appears to be a binding locus close to the carbohydrate binding site of SBA which interacts with a phenyl ring glycosidically linked directly to the sugar, inasmuch as *p*-nitrophenyl β-D-GalNAc*p* was approximately three to four times better as an inhibitor than Me β-D-GalNAc*p* (compare also Poretz and Goldstein, 1971; Pereira et al., 1974).

DB. Relatively little information on contact groups was obtained due to the low sensitivity of the system employed. However, as was found for HP and SBA, the acetamido group at C-2 appears to be in contact with the site. The C-4 hydroxyl (axial) also may be in contact with the site since D-GlcNAc or its methyl α- and β-glycosides were noninhibitors. However, an equatorially oriented hydroxyl at C-4 (as in D-GlcNAc) may destabilize the interaction due to steric hindrance.

LBL. As for DB the low sensitivity of the test system precludes a more detailed analysis. It would, however, appear that the C-2 acetamido group makes a positive contribution to binding. One unique feature observed with LBL was that methyl 2-deoxy-2-(*p*-nitrobenzamido)-α-D-galactopyranoside was a much more potent inhibitor than Me α-D-GalNAc*p* (Galbraith and Goldstein, 1972, Table I), probably indicating a binding locus for the aromatic residue in the vicinity of the carbohydrate binding site.

Specificity Range. Of the four lectins investigated, DB appears to be most specific for D-GalNAc, interacting strongly with both its α- and β-glycopyranosides, only very weakly with α- and β-linked D-galactosyl units, and not at all with other commonly occurring monosaccharides. Since the intrinsic association constant for the interaction between DB and D-GalNAc is probably low (see above), DB may be considered as a specific D-GalNAc reagent with clear preference for α-linked D-GalNAc when used as an end-group reagent in structural studies of carbohydrates. This is also demonstrated in the precipitation experiments (Figure 1; Etzler and Kabat, 1970). The only macromolecules precipitated by DB were those with terminal nonreducing α-linked D-GalNAc end groups. The specificity range may therefore be designated DB (α-D-GalNAc*p* > β-D-GalNAc*p*).

Based on the inhibition experiments, LBL appears to have a somewhat broader specificity than DB inasmuch as D-galactose and its methyl glycosides are relatively potent inhibitors (approximately ten times less active than D-GalNAc). D-GlcNAc and other monosaccharides are, however, essentially noninhibitory. LBL shows a clear preference for α-linked D-GalNAc as compared with β-linked D-GalNAc. The association constant for the interaction between LBL and D-GalNAc is low (1×10^3 M^{-1}). These considerations indicate that macromolecules with terminal, nonreducing sugar residues other than D-GalNAc probably interact too weakly with LBL to give rise to precipitation. The results of the precipitin analyses are in agreement with this interpretation (Figure 1). We therefore designate the specificity range: LBL (α-D-GalNAc*p* > β-D-GalNAc*p*).

As defined by inhibition of precipitation SBA has a specificity range similar to LBL. It interacts strongly with D-GalNAc, and also relatively strongly with D-Gal, but not at all with D-GlcNAc or other monosaccharides. However, in contrast to the other lectins, SBA shows no anomeric specificity. SBA has two combining sites and the K_0 value for the interaction with D-GalNAc is relatively high (3×10^4 M^{-1}). The precipitation experiments (Figure 1; Pereira et al., 1974) show

that all macromolecules with α- and β-linked D-GalNAc as well as most of those with nonreducing α- and β-linked D-galactosyl end groups are precipitated by this lectin. However, those with nonreducing D-GlcNAc end groups are not precipitated. The specificity range may therefore be defined as: SBA (α,β-D-GalNAc*p* > α,β-D-Gal*p*).

HP has the broadest specificity of all the four α-D-GalNAc-binding lectins investigated. It interacts strongly with α-linked D-GalNAc, relatively strongly with α-linked GlcNAc and β-linked D-GalNAc, weakly with β-linked D-GlcNAc, and very weakly with α-linked D-Gal. HP is hexavalent and the K_0 value for the interaction with D-GalNAc is 5×10^3 M^{-1}. Due to its high valency, HP is a very potent precipitinogen and also binds strongly to cells (multipoint binding) (Hammarström, 1974). As can be seen from Figure 1 (compare also Hammarström and Kabat, 1969, 1971; Hammarström et al., 1972), HP precipitated all macromolecules with α- and β-linked D-GalNAc and α-linked D-GlcNAc as well as several of those with β-linked D-GlcNAc and α-linked D-Gal nonreducing end groups. However, macromolecules with terminal nonreducing β-linked D-galactosyl end groups are not precipitated by HP (0/4 examples, Figure 1). The specificity range of HP may therefore be designated: HP (α-D-GalNAc*p* > α-D-GlcNAc $\simeq$ β-D-GalNAc*p* > β-D-GlcNAc*p* > α-D-Gal*p*).

Acknowledgments

We thank Mrs. Anne-Marie Strömberg for skillful technical assistance.

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