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Further Studies on the Binding Characteristics of Rabbit Liver Galactose/*N*-Acetylgalactosamine-Specific Lectin†

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ABSTRACT: The affinity of various carbohydrates for the galactose/*N*-acetylgalactosamine-specific lectin of the rabbit liver was assessed by determining the effect of these carbohydrates on the binding of [^{125}I]asialoorosomucoid (^{125}I-ASOR) by the lectin. To obtain the concentration of the inhibitor that causes 50% reduction in the ^{125}I-ASOR binding (I_{50}), we carried out inhibition assays with fixed concentrations of ^{125}I-ASOR and the purified, detergent-solubilized lectin, while the concentrations of the inhibitors were varied. The concentrations of the ^{125}I-ASOR and the lectin were chosen such that the I_{50} value obtained closely approximates the dissociation constant of the inhibitor. Previously, we had shown that equatorial 2-hydroxyl (or acetamido), equatorial 3-hydroxyl, and axial 4-hydroxyl groups of a D-galactopyranosyl (or 2-acetamido-2-deoxy-D-galactopyranosyl) residue in the neoglycoprotein ligand participate in the binding to the lectin [Stowell, C. P., Lee, R. T., & Lee, Y. C. (1980) *Biochemistry 19*, 4904-4908; Lee, R. T. (1982) *Biochemistry 21*, 1045-1050]. In this study, we demonstrate that the methylene group (C-6) and certain aglycons also contribute to the binding. The presence of an unsaturated group such as C=NH at the γ position to the anomeric carbon enhances the binding of an equatorially oriented aglycon. In addition, there seems to be a nonspecific hydrophobic interaction between some aglycons and the lectin binding site. Thus altogether five groups (aglycon, 2-OH or 2-NHAc, 3-OH, 4-OH, and 6-CH_2–) in a galactopyranoside (or *N*-acetylgalactosaminide) have been shown to participate in lectin–ligand interactions. However, not all five groups are absolutely necessary for binding, since significant binding to the liver lectin occurs when only four of these groups are present.

We have previously investigated the ligand structural requirements of the galactose/*N*-acetylgalactosamine-specific lectin of rabbit liver using bovine serum albumin (BSA)[1] derivative containing various galactose[2] analogues as inhibitors of ^{125}I-ASOR binding to the lectin (Krantz et al., 1976; Stowell et al., 1980). These early experiments were performed with the amidino-type BSA neoglycoproteins having Glyc—$SCH_2C(=NH)NH$—protein structure (Lee et al., 1976). It was found that the BSA derivatives of this type containing either glucose or L-arabinose were as inhibitory as galactose-containing BSA derivatives, implying that the lectin requires neither the axial 4-OH nor the –CH_2OH substituent on the C-5. These results suggested that this lectin might have

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[1] Abbreviations: BSA, bovine serum albumin; ASOR, asialoorosomucoid; TLC, thin-layer chromatography.

[2] All sugars are of the D configuration unless otherwise specified.

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an unusually broad specificity. More recently, however, using several new types of BSA neoglycoproteins having different sugar-to-protein linkages, we found that, in general, glucose derivatives do not bind as well as their galactose counterparts and that the axial 4-OH of galactose contributes significantly to the binding (Lee, 1982). It was concluded that the sugar–protein linkage group, $-CH_2C(=NH)NH-$, in the imidate-modified BSA interacts strongly with the lectin and somehow strengthens the otherwise weak binding of the glucose moiety.

In this study we used a large number of glycosides as inhibitors of the ^{125}I-ASOR–lectin complex formation to study the interaction of the lectin with the C-6 region and the aglycon portion of a galactopyranoside in detail. The results showed that the C-6 methylene group apparently participates in the binding. As is the case for the neoglycoproteins, an amidino group in the aglycon at the γ position from the anomeric carbon interacts strongly with the lectin. This interaction effectively reduces the specificity of the lectin by increasing the association constant of otherwise weakly bound sugars, such as glucose and L-arabinose. Since the desialylated serum glycoproteins lack this type of group in the aglycon of galactose, the "natural" specificity of this lectin is expected to be more stringent than that shown by the amidino-type neoglycoproteins.

Materials and Methods

The following compounds were obtained from the indicated commercial sources: methyl α-D-glucopyranoside, methyl β-D-glucopyranoside, methyl α-D-mannopyranoside, phenyl β-D-glucopyranoside, *p*-nitrophenyl β-D-glucopyranoside, D-galactopyranuronic acid monohydrate, 6-*O*-phospho-D-galactose (Ba^{2+} salt), and 1-*O*-phospho-α-D-galactopyranose ($2K^+$ salt) from Sigma Chemical Co.; cellobiose, maltose hydrate, and melibiose monohydrate from Pfanstiehl Laboratories, Inc.; methyl β-L-arabinopyranoside from Pierce Chemical Co.; 1,2:3,4-di-*O*-isopropylidene-α-D-galactopyranose from Koch-Light Laboratories, Ltd., *p*-toluenesulfonyl chloride, *N*-(ethoxycarbonyl)-2-ethoxy-1,2-dihydroquinoline, triphenylphosphine, carbon tetrabromide, and 10% palladium catalyst on carbon from Aldrich Chemical Co.; Na^{125}I from Amersham Corp. Orosomucoid (a gift from the American Red Cross National Fractionation Center) was desialylated by a mild acid treatment as previously described (Connolly et al., 1981).

All evaporation was done in a rotary evaporator at reduced pressure under 40 °C. Melting points were determined with a Fisher-Johns apparatus. Elemental analyses were performed by Guelph Chemical Laboratories Ltd. (Guelph, Ontario, Canada). Carbohydrate in the column effluent was assayed with a phenol–sulfuric acid method (McKelvy & Lee, 1969). TLC was performed on silica gel layers precoated on aluminum sheets (Merck, silica gel 60, F-254, 0.2 mm) in the following solvent systems: (A) 1:1 (v/v) toluene–ether, (B) 8:1 (v/v) ethyl acetate–acetone, (C) 4:1 (v/v) ethyl acetate–acetone, (D) 1:1 (v/v) ethyl acetate–acetone, (E) 10:1 (v/v) chloroform–methanol, (F) 9:2 (v/v) chloroform–methanol, (G) 1:1 (v/v) chloroform–methanol, (H) 9:4:2 (v/v/v) ethyl acetate–isopropyl alcohol–water, (I) 9:2:1 (v/v/v) ethyl acetate–isopropyl alcohol–water, (J) 3:2:1 (v/v/v) ethyl acetate–isopropyl alcohol–water, (K) 8:2:1 (v/v/v) ethyl acetate–acetic acid–water, (L) 3:2:1 (v/v/v) ethyl acetate–acetic acid–water, (M) 3:2:1 (v/v/v) ethyl acetate–pyridine–water, (N) 5:5:1:3 (v/v/v/v) ethyl acetate–pyridine–acetic acid–water. TLC plates were first viewed under UV light to locate UV-absorbing components, and then the carbohydrates were visualized by spraying with 15% sulfuric acid in 50% ethanol and heating briefly on a hot plate. The primary amino group was detected by spraying the plates with 0.4% ninhydrine in acetone and heating them briefly. Radioactivity (^{125}I) was counted by a Packard Prias PGD auto γ counter.

The affinity of various carbohydrates for the rabbit liver lectin was assessed by measuring at several concentrations the ability of these compounds to inhibit ^{125}I-ASOR binding to the lectin. The inhibition assay, which is based on assay A of Hudgin et al. (1974), has been described (Connolly et al., 1982). Briefly, an inhibitor (usually in the concentration range of 5×10^{-2} to 5×10^{-6} M) and ^{125}I-ASOR ($\sim 6 \times 10^{-10}$ M) were incubated with the lectin ($\sim 4 \times 10^{-10}$ M) in a total volume of 0.5 mL at pH 7.8 in the presence of 0.05 M $CaCl_2$, 0.6% BSA, and 0.5% Triton X-100 for 0.5 h at 25 °C. The lectin was precipitated with 45% ammonium sulfate and collected by filtration through a glass fiber disk (Whatman GF/C). Radioactivity on the disk was counted to determine the amount of ^{125}I-ASOR bound to the lectin. For each inhibitor, the percent inhibition was plotted vs. the log of the inhibitor concentration to yield sigmoidal curves similar to those shown by Van Lenten & Ashwell (1972). The concentration of an inhibitor causing 50% inhibition (I_{50}) was determined from the curve. Since the inhibition assay was performed with concentrations of the lectin and the ligand (^{125}I-ASOR) $\sim$10-fold lower than K_D, these I_{50} values should closely approximate the K_I values (Chang et al., 1975; Jacobs et al., 1975). The binding constant of some glycoside inhibitors was also determined by using the program LIGAND developed by Munson & Rodbard (1980) for computerized curve fitting of ligand-binding systems. By this program, inhibition of ^{125}I-ASOR by unlabeled ASOR yielded the dissociation constant for ASOR of 4.8×10^{-9} M, and this value was held constant in the subsequent analyses of glycoside inhibition data. The I_{50} value for ASOR by the graphic method is $(4.0 \pm 1.5) \times 10^{-9}$ M, in good agreement with the computer-generated K_I value. Dissociation constants of several glycoside inhibitors (K_I) obtained in this fashion lie within the experimental range of I_{50} values much as shown for ASOR, providing further support that I_{50} values obtained under these conditions approximate the dissociation constants. For instance, the I_{50} value for **1** was 0.6 ± 0.2 mM, and K_I of **1** was 0.45 mM.

The following compounds were prepared according to the published methods: allyl β-D-galactopyranoside and allyl β-D-glucopyranoside (Lee & Lee, 1974); carboxymethyl 1-thio-β-D-galactopyranoside and carboxymethyl 1-thio-β-D-glucopyranoside (Krantz et al., 1976); cyanomethyl 1-thiopyranosides and their per-*O*-acetylated derivatives (Lee et al., 1976; Stowell & Lee, 1980); [[(2,2-dimethoxyethyl)amino]-carbonyl]methyl 1-thioglycopyranosides and [[(2,2-dimethoxyethyl)amino]carbonyl]pentyl 1-thioglycopyranosides (Lee & Lee, 1979a); β-Gal(1→4)GlcNAc (Lee & Lee, 1979b); β-GlcNAc(1→6)Gal (Lee & Lee, 1982); β-Gal(1→3)GlcNAc and β-Gal(1→6)GlcNAc (Flowers, 1978); 6-aminohexyl glycosides of β-Gal(1→3)β-GlcNAc, β-Gal(1→4)β-GlcNAc and β-Gal(1→6)β-GlcNAc (Vernon et al., 1980); methyl α-D-fucopyranoside (MacPhillamy & Elderfield, 1939). 1,2:3,4-Di-*O*-isopyropylidene-6-*O*-tosyl-α-D-galactopyranose was prepared from 1,2:3,4-di-*O*-isoprylidene-α-D-galactopyranose by a modification of the method described by Petit et al. (1980). The crystalline product was obtained in 75% yield; mp 98–100.5 °C [lit. (Freudenberg & Raschig, 1927) mp 102–103 °C], homogeneous by TLC in solvent A. This product was deacetalized with 90% trifluoroacetic acid according to the general method described by Christensen &

Goodman (1968) to yield 6-*O*-tosyl-β-D-galactopyranose in 76.6% yield: homogeneous by TLC in solvent K; mp 129 °C (with decomposition) [lit. (Ohle & Thiel, 1933) mp 130 °C (decomposition)]. Methyl α-D-galactopyranoside monohydrate was tosylated by a modification of the method of Haworth et al. (1940) with *p*-toluenesulfonyl chloride in dry pyridine in the presence of 4-Å molecular sieves. The crystalline product was obtained in 54% yield: homogeneous by TLC in solvents D, E, and I; mp 156–158 °C (decomposition) [lit. (Ohle & Thiel, 1933) mp 170 °C]. Methyl 3,6-anhydro-α-D-galactopyranoside was prepared according to the method of Haworth et al. (1940): mp 138–139 °C [lit. (Ohle & Thiel, 1933) mp 140 °C]. Methyl 4,6-*O*-benzylidene-α-D-galactopyranoside was prepared by the method of Sorkin & Reichstein (1946): mp 167–177 °C (lit. mp 177–178 °C).

Methyl (methyl α-D-galactopyranosid)uronate monohydrate was prepared from galacturonic acid monohydrate by the method of Jones & Stacey (1947): mp 145–146 °C, (lit. mp 147 °C); homogeneous by TLC in solvents G and L. Methyl α-D-galactopyranosiduronic acid dihydrate was prepared from the methyl ester by the method of Morell & Link (1933) without isolation of the intermediate barium salt. The product was homogeneous by TLC in solvents L, M, and N; mp 86–89 °C (lit. mp 112–114 °C). Treatment of the methyl ester with liquid ammonia at −70 °C for 0.75 h, followed by evaporation of the excess ammonia at room temperature, resulted in formation of the solid methyl α-D-galactopyranosiduronamide in 82% yield; homogeneous by TLC in solvents G, J, and L. The hydrazide of methyl α-D-galactopyranosiduronic acid was also prepared from the corresponding methyl ester by treating it with hydrazine monohydrate for 18 h at room temperature. After thorough removal of the hydrazine by azeotropic evaporation with toluene, a crystalline product was obtained in 91%. The crystallized material was homogeneous by TLC in solvents, J, M, and N. β-D-Glucopyranosyl—$SCH_2C(=NH)NHCH_2CO_2H$ (**1**) was prepared by reacting 2-imino-2-(methoxyethyl)-1-thio-β-D-glucopyranoside with glycine according to the published method for the γ-aminobutyric acid analogue (Lee et al., 1976); mp 145–148 °C (decomposition). β-D-Galactopyranosyl—$SCH_2C(=NH)NHCH_2CO_2H$ (**2**) was prepared similarly; mp 158–160 °C. Methyl 2-*O*-acetyl-α-D-galactopyranoside was prepared according to the method of R. Kaifu and I. J. Goldstein (private communication) from methyl 3,4-*O*-isopropylidene-6-*O*-trityl-α-D-galactopyranoside (kindly provided by Drs. R. Kaifu and I. J. Goldstein). Preparation of the following compounds will be published elsewhere: 1-cyano-1-deoxy-β-D-galactopyranose and 1-carboxyl-1-deoxy-β-D-galactopyranose (R. W. Myers and Y. C. Lee, unpublished results). The rabbit liver lectin was prepared essentially according to the method of Hudgin et al. (1974), with asialofetuin–Sepharose 4B as affinant.

Results

Methyl 6-Azido-6-deoxy-α-D-galactopyranoside. A solution containing vacuum-dried methyl 6-*O*-tosyl-α-D-galactopyranoside (1.74 g, 5 mmol) and sodium azide (1.30 g, 20 mmol) in 25 mL of dry *N*,*N*-dimethylformamide was heated at 120 °C for 48 h with stirring. The mixture was evaporated under vacuum to a syrup, which was suspended in 40 mL of acetone. Insoluble material was removed by filtration, and the filtrate was evaporated again to a syrup. The syrup was chromatographed on a column (2 × 40 cm) of silica gel with solvent B as eluant. The material in the first carbohydrate peak was isolated in 18% yield as crystals from 95% ethanol and was identified as methyl 3,6-anhydro-α-D-galactopyranoside by comparing it (mp and TLC) with the material synthesized according to the method of Haworth et al. (1940). From the second carbohydrate peak methyl 6-azido-6-deoxy-α-D-galactopyranoside (0.26 g, 1.19 mmol, 24%) was obtained as crystals from 95% ethanol: mp 176–177 °C [lit. (DeJongh & Hanessian, 1965) mp 172–173 °]; homogeneous by TLC with solvent C.

Methyl 6-Amino-6-deoxy-α-D-galactopyranoside. The 6-azido derivative (0.15 g, 0.7 mmol) in 3.35 mL of dry methanol was hydrogenated in the presence of 10% palladium on carbon (25 mg) in a micro Brown hydrogenator (Brown & Brown, 1966). After 10 h the catalyst was removed by filtration, and the evaporation of the filtrate produced the title compound as syrup, which gave a single spot detectable both by charring and by ninhydrin in TLC with solvents L and N.

Methyl 6-Bromo-6-deoxy-α-D-galactopyranoside. Selective bromination of methyl α-D-galactopyranoside was carried out by a modification of the general method described by Anisuzzman & Whistler (1978). A solution of methyl α-D-galactopyranoside monohydrate (1.06 g, 5 mmol) in 50 mL of dry pyridine was stirred for 2 h with 4-Å molecular sieves (~5 g) at room temperature and cooled to 0 °C. To this solution was added triphenylphosphine (2.89 g, 11 mmol), followed by carbon tetrabromide (1.82 g, 5.5 mmol). The solution was stirred for 0.25 h at 0 °C, 0.75 h at 55°, and then 2 days at room temperature. After addition of 1 mL of methanol, the solution was heated for 0.25 h at 55 °C and evaporated under vacuum. The resulting syrup was partitioned between chloroform and water (50 mL each). The chloroform phase was washed twice with 50 mL of water, and the combined aqueous phase was washed with chloroform and ether (25 mL each). The aqueous phase was adjusted to pH 7 with NaOH and evaporated to a small volume. The pH was then readjusted and the remaining liquid was removed by evaporation. The resulting syrup was dissolved in 10 mL of 95% ethanol and fractionated on a column (5 × 194 cm) of Sephadex LH-20 in 95% ethanol. Elution with 95% ethanol (collecting 20 mL/fraction) produced three carbohydrate peaks, which were, in the order of elution, unreacted methyl α-D-galactopyranoside (fractions 142–147), methyl 3,6-anhydro-α-D-galactopyranoside (fractions 148–157), and methyl 6-bromo-6-deoxy-α-D-galactopyranoside (fractions 159–169). The last glycoside was obtained as crystals upon concentration of fractions 159–169: yield 0.25 g, 0.97 mmol (19.4%); mp 152.5–154.5 °C (decomposition) [lit. (Hanessian & Plessas, 1969) mp 174–175 °C]; homogeneous by TLC in solvents D, G, and J. This product was identical with 6-bromo-6-deoxy-α-D-galactopyranoside prepared from methyl 4,6-*O*-benzylidene-α-D-galactopyranoside via methyl 4-*O*-benzoyl-6-bromo-6-deoxy-α-D-galactopyranoside according to the method of Hanessian & Plessas (1969).

β-D-Glucopyranosyl-$SCH_2CONHCH_2CO_2Et$ (**3**) *and β-D-Galactopyranosyl-$SCH_2CONHCH_2CO_2Et$* (**4**). These compounds were prepared from carboxymethyl 1-thioglycoside and glycine ethyl ester hydrochloride. *N*-(Ethoxycarbonyl)-2-ethoxy-1,2-dihydroquinoline was used as the activator of the carboxylic acid in these reactions according to the method of Barker et al. (1974). The reaction mixture was purified by passing it through a column (5 × 220 cm) of Sephadex G-25 in 0.1 M acetic acid. Fractions of the major peak that contained the desired product (**3** or **4**) were combined and evaporated, and the product was crystallized from hot ethyl acetate. The yield of crystalline **3** was 0.31 g (0.9 mmol, 45% yield); mp 100–103 °C homogeneous in TLC (R_f 0.64 in solvent J). The yield of crystalline **4** was 0.29 g (0.85 mmol, 43% yield): mp 120–124 °C; homogeneous in TLC (R_f 0.55

Table I: I_{50} Values of Analogues of Methyl α-D-Galactopyranoside Having Different Substituents at C-5

C-5 substituent	I_{50} (mM)	C-5 substituent	I_{50} (mM)
CH_2-OH (Gal)[a]	1.6	H (L-Ara)	12
CH_2-H (D-Fuc)	1.6	CO_2Me	16
CH_2-N_3	1.2	$CONH_2$	19
CH_2-Br	1.1	$CONHNH_2$	9
CH_2-NH_2	4.3	CO_2H	~200
CH_2-NH_2[b]	1.9		
CH_2-OTs[c]	1.0		

[a] The I_{50} value is from Connolly et al. (1982). [b] Methyl glycoside of β configuration. [c] Due to unsufficient solubility of the methyl glycoside, the reducing sugar (6-*O*-tosyl-D-galactose) was used.

Table II: I_{50} Values of Various β-Glycosides of D-Galactopyranose and D-Glucopyranose

aglycon	I_{50} (mM) Glc[a]	I_{50} (mM) Gal[a]
none	16	1.7[b]
OCH_3[c]	26	1.6[b]
OCH_3	17	1.0[b]
$OCH_2CH{=}CH_2$	12	0.47
$O(CH_2)_6NH_2$		0.28[b]
SCH_2CO_2H	12	2.0
$SCH_2C{\equiv}N$	2.0	0.13
$SCH_2C(=NH)NH_2$	0.8 (6)	0.3 (7)
OPh	4.0	
$OC_6H_4NO_2$ (para)	1.7	
$SCH_2CONHCH_2CO_2Et$	2.5 (3)	0.7 (4)
$SCH_2CONHCH_2CO_2H$	2.1 (5)	
$SCH_2C(=NH)NHCH_2CO_2H$	0.75 (1)	0.6 (2)
$SCH_2CONHCH_2CH(OMe)_2$	2.0	0.75
$S(CH_2)_5CONHCH_2CH(OMe)_2$	4.3	0.23
(1,4)Glc	2.0 (cellobiose)	0.3 (lactose)
(1,4)Glc[c]	2.6 (maltose)	
(1,4)GlcSCH$_2$C≡N	1.5 (cyanomethyl thiocellobioside)	

[a] Name or number of the compound is given in parentheses. [b] Data from Connolly et al. (1982). [c] α-Glycoside.

in solvent J). Anal. of **3**. Calcd for $C_{12}H_{21}NO_8S$ (339.25): C, 42.45; H, 6.23; N, 4.12; S, 9.45. Found: C, 42.35; H, 6.22; N, 4.02; S, 9.51.

An aqueous solution of **3** was adjusted and maintained at pH 11.5 by the addition of NaOH. When the pH of the solution no longer drifted downward (~0.5 h), the solution was neutralized to pH 7 with Dowex 50X8 (H^+ form) and filtered and the filtrate evaporated to yield the sodium salt of β-glucopyranosyl-$SCH_2CONHCH_2CO_2H$ (**5**) as a white solid.

Amidinomethyl 1-Thio-β-D-glucopyranoside (**6**) *and Amidinomethyl 1-Thio-β-D-galactopyranoside* (**7**). These compounds were prepared according to the method of Schaefer & Peters (1961). 2-Imino-2-methoxyethyl 1-thio-β-D-glucopyranoside was generated from the corresponding cyanomethyl per-*O*-acetyl-1-thio-glycoside in methanol containing sodium methoxide. The base was neutralized with solid CO_2, and NH_4Cl was added in 30% molar excess over the imidate. The reaction mixture consisted of the product and the deacetylated cyanomethyl 1-thioglycoside in addition to some inorganic salts. The product was partially separated from the cyanomethyl thioglycoside by passing the mixture through a column (5 × 220 cm) of Sephadex G-25 in 0.1 M acetic acid. Fractions containing mainly the amidino derivative were combined and evaporated, and the acetate salt of **6** was crystallized from water–ethanol. The yield was 30% from cyanomethyl per-*O*-acetyl-1-thio-β-D-glucopyranoside: mp 160–168 °C (decomposition); homogeneous by TLC in solvent N. The corresponding galactose derivative (**7**) was prepared similarly; mp 151–156 °C. Anal. of the acetate salt of **6**. Calcd for $C_{10}H_{20}N_2O_7S$ (312.25): C, 38.43; H, 6.45; N, 8.97; S, 10.26. Found: C, 38.52; H, 6.63; N, 8.97; S, 10.13.

Inhibition Assay. Plots of percent inhibition of ^{125}I-ASOR binding vs. the log of the inhibitor concentration exhibited sigmoid curves as observed by Van Lenten & Ashwell (1972). The slope of these curves were remarkably similar. In Tables I–IV are presented the I_{50} values for each compound obtained from the sigmoidal curves. An I_{50} value higher than 50 mM is usually an approximation, since often only a partial inhibition curve could be generated.

The I_{50} values of derivatives of methyl α-D-galactopyranoside modified at C-6 are presented in Table I. Comparison of the right and left panels shows that I_{50} values of all the compounds possessing a C-6 methylene group bind ~10-fold more strongly than those lacking the methylene group. As observed with neoglycoprotein inhibitors (Lee, 1982), oxidation of C-6 to carboxylic acid decreases the binding greatly. However, the presence of an amino group at this position had no effect or only a slight effect on the I_{50} value.

Table II presents the effect of various aglycons of gluco- and galactopyranosides on the binding strength. The following observations were made: (1) In general, aglycons having a chain length of three atoms or longer increased the binding power of both galactose and glucose. The effect is usually more pronounced in glucosides than in the corresponding galactosides. (2) Aglycons of diverse structures such as aminohexanol and glucose lowered the I_{50} value to a similar extent, suggesting that there is a nonspecific and possibly hydrophobic interaction between many of the aglycons and the lectin. (3) The presence of C=N or C≡N group on the third atom from the anomeric carbon had a specific effect of increasing the binding strength even when the aglycon length is short. This effect is more pronounced in glucosides than in galactosides.

The effect of the aglycon on the binding of sugars other than galactose and glucose is shown in Table III. Aglycons had little or no effect on mannose and *N*-acetylglucosamine, while an interacting aglycon strengthened the binding of L-arabinose and methyl galacturonate ~10-fold, as observed with glucose derivatives.

The following observations were made from the data presented in Table IV: (1) All disaccharides and their gly-

Table III: Effect of Equatorial Aglycons on I_{50} of Sugars Other Than Glucose and Galactose

aglycon	I_{50} (mM) Man	GlcNAc	L-Ara	methyl galacturonate	sodium galacturonate
none	45	~100			
OCH_3 (α)	50		12	16	~200
SCH_2CN	14	70	1.2		
$SCH_2CONHCH_2CH(OMe)_2$	55	~100		1.2	7.6
$S(CH_2)_5CONHCH_2CH(OMe)_2$	45				

Table IV: I_{50} Values of Miscellaneous Sugars and Glycosides

compound	I_{50} (mM)
Galβ(1→3)GlcNAc	0.9
Galβ(1→4)GlcNAc	0.8
Galβ(1→6)GlcNAc	0.3
Galβ(1→3)GlcNAcO$(CH_2)_6NH_2$ (β)	0.2
Galβ(1→4)GlcNAcO$(CH_2)_6NH_2$ (β)	0.4
Galβ(1→6)GlcNAcO$(CH_2)_6NH_2$ (β)	0.5
Galβ(1→6)Glc	0.9
Galα(1→6)Glc	2.8
GlcNAcβ(1→6)Gal	2.8
GlcNAcβ(1→6)Gal-$SCH_2CONHCH_2CH(OMe)_2$ (β)	0.6
N-acetylgalactosamine[a]	0.09
Me 2-*O*-Ac-α-Gal	0.04
1-cyano-1-deoxy-Gal (β)	1.0
1-carboxyl-1-deoxy-Gal (β)	2.5
6-*O*-phospho-Gal (Ba^{2+} salt)	35
1-*O*-phospho-α-Gal ($2K^+$ salt)	22
Me 3,6-anhydro-α-Gal	≥180[b]
Me 4,6-*O*-benzylidene-α-Gal	≥20[b]

[a] From Connolly et al. (1982). [b] Not inhibitory at the concentrations shown.

cosides having a β-D-galactopyranosyl residue as the nonreducing end were more inhibitory than galactose and had I_{50} values in the range of 0.2–0.9 mM regardless of the nature of the second sugar or the linkage position. (2) The lectin can tolerate the presence of a bulky substituent such as *N*-acetylglucosaminyl and tosyl groups (Table I) at C-6 of galactose. (3) Attachment of an acetyl group to 2-OH of methyl α-galactopyranoside strengthened the inhibitory power 40-fold, making it as inhibitory as its 2-acetamido-2-deoxy analogue, *N*-acetylgalactosamine. (4) The 1-O- and 6-O-phosphorylated derivatives of galactose were noninhibitory. A carboxylic acid (a weaker acid than phosphate) on or near C-1 lowered the binding strength only slightly (see also Table II). The presence of a carboxylic acid on the aglycon at a position distant from the anomeric carbon does not appear to affect the binding constant (compare **3** and **5** in Table II). (5) The two derivatives of methyl α-galactopyranoside having a second fused ring did not show any inhibition even at the highest concentratioins tested.

Discussion

We have investigated sugar specificity and other binding characteristics of the rabbit liver galactose/*N*-acetylgalactosamine-specific lectin using neoglycoproteins bearing various sugars as inhibitors of ^{125}I-ASOR binding to the lectin (Krantz et al., 1976; Stowell et al., 1980; Lee, 1982). Though most of the studies were carried out with neoglycoproteins of BSA derivatized with thioglycosides, the specificity expressed by these BSA derivatives agreed well with the results obtained with derivatives of serum glycoproteins (Van Lenten & Ashell, 1972; Kawasaki & Ashwell, 1977; Sarkar et al., 1979). We found that BSA derivatives containing galactose and *N*-acetylgalactosamine were good inhibitors and their inhibitory power increased exponentially with the sugar content, while BSA neoglycoproteins containing mannose and *N*-acetylglucosamine were not inhibitory (tested at $\sim 5 \times 10^{-5}$ M BSA) even at the highest sugar content (~40 mol/mol of BSA). Therefore, the higher the sugar content, the more pronounced becomes the difference in the inhibitory effect of the galactose–BSA compared to that of the corresponding mannose (or *N*-acetylglucosamine)–BSA. For example, a BSA derivative containing 25 mol of galactose/mol is a potent inhibitor, equivalent to ASOR in both inhibitory power and sugar content on a weight basis (~3.8 mol of sugar/10^4 daltons), whereas the binding strength of the corresponding mannose–BSA is estimated to be at least 10^4-fold weaker.

The lectin appears to recognize only a small portion of the glycoprotein ligand, the galactose (or *N*-acetylgalactosamine) moiety being the major structural feature necessary for binding (Sarkar et al., 1979). This conclusion is further reinforced by the observation that all the neoglycoproteins having a galactose content of ~3.8 mol/10^4 daltons are as inhibitory as ASOR regardless of the nature of protein or the linking group between the sugar and the protein (Lee & Lee, 1980; Lee, 1982).

Although neoglycoprotein inhibitors allow a clear distinction between binding and nonbinding species by magnifying their differences, the preparation of the neoglycoproteins is considerably more tedious than the preparation of simple glycosides. Therefore, in this study a large number of glycosides, rather than neoglycoproteins, were tested.

In an earlier study using sugars and glycosides as inhibitors of ASOR binding to the rabbit liver lectin (Connolly et al., 1982), it was found that (i) the glycosyl O can be replaced by S with little change in I_{50}, (ii) the anomeric configuration of a linear aglycon had no effect on I_{50}, and (iii) a large aglycon such as a 6-aminohexyl group apparently interacted with lectin to increase the binding strength of galactose ~4-fold. This interaction between aglycon and the lectin was also observed earlier by Sarkar et al. (1979). In our present study it was found that a large number of galactopyranosides had I_{50} values in the range of 0.2–0.9 mM compared to the parent sugar, galactose, whose I_{50} value was 1.7 mM (Table II), indicating that aglycons of diverse structure strengthen the binding of galactose to the lectin by approximately 4-fold. Mannopyranosides as well as mannose, on the other hand, had I_{50} values of ~45 mM (Table III). Since galactose and mannose in the desialylated, serum glycoproteins and neoglycoproteins represent good binding and nonbinding species, respectively, those glycosides having I_{50} values lower than 0.9 mM in this study were defined as "strongly binding" glycosides and those with I_{50} values higher than 45 mM as "nonbinding" glycosides. The intermediate I_{50} values perhaps indicate ability to bind weakly to the lectin.

As seen in Table II, in general, aglycons that enhance the binding of glycosides showed a greater effect on glucose (I_{50} = 16 mM) than on galactose (I_{50} = 1.7 mM), such that the I_{50} values of glucopyranosides were only slightly higher than those of corresponding galactopyranosides. In our earlier studies with neoglycoprotein inhibitors, it was observed that, in contrast to the galactose-containing BSA derivatives, the inhibitory power of the glucose-containing BSA derivatives varied considerably depending on the sugar-to-protein linkage group (Lee, 1982). All the glucose–BSA derivatives except one were poorer inhibitors than the galactose–BSA derivatives except one were poorer inhibitors than the galactose–BSA derivatives. The exception was glucose–BSA, having a linking structure of Glc—$SCH_2C(=NH)NH$—BSA (Glc-AI-BSA), which was almost as inhibitory as the galactose counterpart. This observation led us to conclude that a strong interaction existed between the lectin and the amidino group located at the γ position from the anomeric carbon. A somewhat weaker interaction was also observed between the lectin and C=O group at the same position.

In the present study we found that the two most inhibitory glucosides both had a common aglycon structure of Glc—$SCH_2C(=NH)NH$— and that amongst all the glucosides tested only these two glucosides had binding strength comparable to that of galactosides (Table II). Curiously, this amidinomethyl group strengthened the binding of glucose

Table V: Comparison of I_{50} of Glycosides Having Three and Four Binding Groups[a]

nonbinding glycosides (three binding groups)			weakly binding glycosides (four binding groups)		
glycosides	binding groups missing at	I_{50} (mM)	glycosides	binding group missing at	I_{50} (mM)
Me β-Glc	C-1, C-4	17	Glc-SCH_2CN	C-4	2.0
Me β-L-Ara	C-1, C-6	12	L-Ara-SCH_2CN	C-6	1.2
Me (Me α-galactosid)uronate	C-1, C-6	16	$SCH_2CONHCH_2CH(OMe)_2$ glycoside of Me galacturonate	C-6	1.2
Man-SCH_2CN	C-2, C-4	14	Me β-Gal	C-1	1.0

[a] Glycoside inhibitors with five binding groups had $I_{50} < 0.9$ mM.

20-fold, while increasing the strength of galactose only by 3–6-fold. As shown in Table II, many of other glucosides had I_{50} values of 1.5–4.3 mM, which was only 2–6-fold higher than the potent inhibitors **1** and **6**. However, this slight lowering in affinity apparently brings about a significant difference in the binding power when these glucosides are in the form of neoglycoprotein. For instance, as a monovalent ligand, the inhibitory power of cellobiose or its glycoside (I_{50} of 1.5–2 mM) was only slightly weaker than that of amidinomethyl thioglucoside (0.8 mM). Yet in the form of a multivalent neoglycoprotein, BSA containing 25 mol/mol of cellobiose (cellobiose–AI–BSA) was an $\sim 10^3$-fold poorer inhibitor than Glc–AI–BSA (the amidino linked) of the same sugar content (Lee, 1982). It seems that the I_{50} value of ~ 1 mM represents a threshold binding strength for tight binding to occur between a multivalent ligand (neoglycoprotein) and the lectin (which may also exist in multivalent states). A similar phenomenon of threshold binding constant for tight binding has been observed in the interaction of glycopeptides with a lectin that was in a multivalent state on an affinity medium (Baenziger & Fiete, 1979).

The results shown in Table I indicate that the C-6 methylene group contributes to binding. For instance, methyl L-arabinoside and methyl (methyl galactopyranosid)uronate, both of which lacked the methylene group, were nonbinders. These results appear to contradict the earlier inhibition studies with neoglycoproteins (Stowell et al., 1980; Lee, 1982) in which it was shown that the C-5 substituent (CH_2OH) can be absent (i.e., L-arabinose) or can be modified with a large group, suggesting a total lack of specificity of the lectin for this position. However, just as in the case of glucose, the presence of a strongly interacting aglycon strengthened the binding of these sugars almost to the level of the galactosides (Table III). Therefore, these strongly interacting aglycons had a net effect of reducing the structural requirements of the lectin. In fact, earlier specificity studies using exclusively the amdino-type neoglycoproteins indicated the lectin has very broad specificity, requiring only the correct orientation of the 2-OH and 3-OH groups of a galactosyl residue (Stowell et al., 1980). Since natural glycoproteins lack this strongly interacting amidino group, the "natural" specificity of the lectin is expected to be more refined, recognizing the axial 4-OH and the C-6 methylene group in addition to 2-OH (equatorial) and 3-OH (equatorial).

Earlier studies have shown that BSA derivatives containing galacturonic acid were very poor inhibitors, while BSA derivatives containing methyl galacturonate were only a little less inhibitory than those containing galactose (Lee, 1982). This led us to postulate that the lectin possesses one or more negatively charged groups in the ligand binding site, which repels galactose analogues having a negative charge at or near the C-6 position, and that this repulsion is directly responsible for the inability of native (sialylated) serum glycoproteins to bind to the lectin. This notion is further strengthened in this study, since the presence of such a negative charge decreased the binding power more than 10-fold (compare methyl glycosides of galacturonic acid and of methyl galacturonate), while a positively charged amino group had little effect. The fact that the positive charge (amino group) does not increase the binding by charge interaction as well as the fact that the C-6 methylene group contributes to the binding suggests that the environment in this region of the lectin is rather hydrophobic so as to discourage all the charged molecules from approaching. The repulsion of the carboxylic acid group appears to be position specific as well, since the presence of a carboxylic acid at other locations does not cause such a drastic decrease in the binding strength (Tables II and IV). The presence of a phosphate group at C-6 and C-1 caused at least a 13-fold decrease in the binding power. Since the binding area of the lectin for the C-6 region of galactose is rather spacious (Lee, 1982), the inhibitory effect of the 6-*O*-phospho group is probably due to its negative charge(s), just as was observed for the 6-COOH group. In the case of the 1-*O*-phospho group, there may be a steric interference by an α-oriented, bulky group (Lee, 1982) in addition to the effect of the negative charge(s).[3]

From the combined results obtained previously (Stowell et al., 1980; Lee, 1982) and in this study, we have identified five groups [aglycon, 2-OH (equatorial),[4] 3-OH (equatorial), 4-OH (axial), and 6-CH_2–] in a galactopyranoside that participate in the binding to lectin. The I_{50} value of <0.9 mM is attained when all five groups are present. As shown in Table V, the presence of four of the five groups is sufficient to bring about weak binding (I_{50} of 1–2 mM), while the presence of only three groups results in a further $\sim$10-fold increase in I_{50} and hence a very poor inhibitor. The only exceptions to this are the two phosphorylated galactoses and glycosides of *N*-acetylglucosamine and galacturonic acid, all of which were nonbinding or very weakly binding in spite of having four binding groups. Results in Table III show that the presence of a strongly interacting aglycon did not improve the binding strength of *N*-acetylglucosamine. As postulated earlier (Lee, 1982), an *N*-acetylglucosaminyl moiety does not bind to the lectin probably due to a steric hindrance. The presence of the equatorial 2-acetamido group in *N*-acetylglucosamine apparently forces this sugar to be bound in a very restrictive way, which in turn results in a strong steric interference by the equatorial 4-OH group. Glucose, on the other hand, having equatorial a 2-OH that interacts with the lectin $\sim$20-fold less strongly than the equatorial 2-acetamido group (Table IV)

[3] Ammonium chloride, potassium phosphate, and sodium acetate were not inhibitory at concentrations comparable to those of charged glycosides. Therefore, any inhibition exhibited by these glycosides is not due to general ionic effects.

[4] The equatorial 2-OH has been shown to contribute to the binding (Stowell et al., 1980). However, as shown in Table IV, the presence of an acetyl group on the equatorial 2-OH or 2-amino group (i.e., *N*-acetylgalactosamine) further strengthens the binding power considerably.

may be allowed to fit in the binding site loosely. In this less rigid state of binding, the steric interference exerted by the equatorial 4-OH is considerably weaker, so that it can be overcome by the presence of a strongly interacting aglycon. Unlike the case of *N*-acetylglucosamine, repulsion of the negatively charged galacturonic acid (I_{50} of ~200 mM) is largely overcome by the presence of an interacting aglycon (Table III). However, the resulting I_{50} value (7.6 mM) is still considerably higher than that necessary for tight binding to occur.

Of all the compounds tested, only two gave no measurable inhibition even at the highest concentrations tested: methyl 3,6-anhydro-α-galactopyranoside at 0.18 M and methyl 4,6-*O*-benzylidene-α-galactopyranoside at 0.02 M (Table IV). Both compounds have a second ring fused almost perpendicular to the pyranose ring. At least for the benzylidene derivative, which is known to be in the normal chair conformation (Baggett et al., 1965), the binding is apparently sterically prohibited by this second ring. The binding of the anhydro derivative would involve a further complication due to the alternate ($^1C_4$) conformation dictated by the second ring. It is not difficult to understand, therefore, that the oxidation of C-6 of a galactosyl residue to an aldehyde by galactose oxidase would abolish the binding completely (Ashwell & Morell, 1974), if the new aldehyde group would form a second ring via an intramolecular hemiacetal linkage (Theander, 1962).

Finally, the anomeric oxygen or sulfur probably does not participate in a nonbonded interaction through the unpaired electrons, since replacement of the oxygen or the sulfur with a carbon (1-cyano-1-deoxy β-D-galactopyranose in Table IV) has little effect on the I_{50} value.

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