Binding of *N*-Acetylgalactosamine-Specific Lectins to Spin-Labeled Galactosamine Derivatives†

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Received January 9, 1986; Revised Manuscript Received February 21, 1986

ABSTRACT: Legume seed lectins specific for *N*-acetyl-α-D-galactosaminyl end groups from *Amphicarpaea bracteata*, lima bean, *Griffonia simplicifolia*, *Dolichos biflorus*, and soybean were compared with respect to binding of several spin-labeled derivatives of D-galactosamine by electron spin resonance and precipitin inhibition analysis. Spin-label II [methyl 2-[[(2,2,5,5-tetramethyl-1-oxopyrrolidin-3-yl)carbonyl]-amino]-2-deoxy-α-D-galactopyranoside], spin-label III [1-(methyl 2-deoxy-α-D-galactopyranosid-2-yl)-3-(2,2,6,6-tetramethyl-1-oxypiperidin-4-yl)-2-thiourea], and spin-label IV [1-[4-[[(methyl 2-deoxy-α-D-galactopyranosid-2-yl)amino]carbonyl]phenyl]-3-(2,2,6,6-tetramethyl-1-oxypiperidin-4-yl)-2-thiourea] contain 2-*N*-(oxypiperidinyl) or 2-*N*-(oxypyrrolidinyl) substituents varying in length and polarity of the linker arm between the glycoside and nitroxide ring. Spin-labels II and III were found to bind very weakly to all the lectins tested ($K_d \geq 1.0$ mM). Spin-label IV, containing a planar, nonpolar 2-*N*-phenyl group, was bound very strongly ($K_d = 0.1$–0.4 mM) and was moderately immobilized ($2T_{\parallel} = 48$–56 G) by all lectins except that from *D. biflorus*. Notably, the affinity of spin-label IV to lima bean lectin was 18-fold greater than that for methyl *N*-acetyl-α-galactosaminide. These results suggest that when the bulky oxypiperidinyl moiety lies in a position close to the sugar ring, it interferes with binding; in the cases where a phenyl group spacer exists, the aromatic ring in some cases actually enhances binding. Unexpectedly, spin-label Ia [1-[4-[(*N*-acetyl-α-D-galactosaminyl)oxy]phenyl]-3-(2,2,6,6-tetramethyl-1-oxypiperidin-4-yl)-2-thiourea] did not appear to bind to any of the lectins tested. Spin-label Ib, the β-anomer, did bind and was moderately immobilized by the *G. simplicifolia* I-A_4 isolectin ($K_d = 0.27$ mM; $2T_{\parallel} = 49$ G) and *A. bracteata* ($K_d = 0.14$ mM; $2T_{\parallel} = 49$ G). Comparison with previous hapten inhibition data suggests again that the oxypiperidinyl group in the α-anomeric configuration sterically blocks binding but does not do so in the β-anomeric form.

The nature of carbohydrate-specific combining sites of plant lectins is of current interest in the context of their development as analytical tools and in discovering a possible physiological role for these proteins. The carbohydrate binding specificity of plant lectins has generally been defined by the ability of simple saccharides to inhibit precipitation of larger molecules or agglutination of cells. As more complex carbohydrates have been examined, lectin–sugar interactions have been shown to be far more complex than anticipated. Extended binding sites have been identified in several lectins, including wheat germ agglutinin (Allen et al., 1973; Wright, 1980), concanavalin A (Brewer et al., 1985), potato lectin (*Solanum tuberosum*; Allen et al., 1973), and *Phaseolus vulgaris* leukoagglutin (Kornfeld & Kornfeld, 1970; Kaifu & Osawa, 1976; Hammarström et al., 1982). Additionally, certain sugar derivatives containing hydrophobic groups have been shown to enhance binding of the hapten, often dramatically, as in the case of concanavalin A (Poretz & Goldstein, 1971), and soybean agglutinin (DeBoeck et al., 1984).

Electron spin resonance (ESR)[1] using spin-labeled glycosides lends itself to studies of carbohydrate–protein interaction in several ways: (1) it provides a direct method of quantitating sugar binding; (2) it affords information on protein–carbohydrate interactions at the molecular level (Goldstein et al., 1985) since the nitroxide moiety can be placed both at specific positions on the glycoside ring and at different distances from the sugar molecule; (3) it potentially allows determination of the distance between sites on the protein, e.g., metal to carbohydrate and carbohydrate to hydrophobic sites, by virtue of spin–spin interactions.

In this paper, we compare several *N*-acetyl-D-galactosamine-specific legume lectins, which differ widely in their affinitites for this sugar, for their interaction with various spin-labeled α-D-galactosaminides.

Materials and Methods

Lectins. Lima bean lectin (LBL) (Roberts et al., 1982) and *Griffonia simplicifolia* isolectins I-A_4 and I-B_4 (Delmotte & Goldstein, 1980) were prepared as described previously. *Amphicarpaea bracteata* seed lectin was purified by affinity chromatography on Synsorb A from Chembiomed (Edmonton, Alberta) and elution with 0.1 M methyl *N*-acetyl-D-galactosamine (Me-α-GalNAc). *Dolichus biflorus* seed lectin was generously provided by Dr. M. E. Etzler (University of California, Davis) and soybean agglutinin (SBA) by Dr. E. Chu of E. Y. Laboratories (San Mateo, CA). PBS buffer consisted of 0.1 M sodium phosphate, 0.15 M NaCl, and 0.1 mM $CaCl_2$, pH 7.1. Lectin concentrations were determined spectrophotometrically with $E^{1\%}_{280}$ and subunit molecular

† This research was supported in part by Grants HD 17270 (L.J.B.) and GM 29470 (I.J.G.) from the U.S. Public Health Service.

[1] Abbreviations: ESR, electron paramagnetic resonance; LBL, lima bean lectin; Me-α-GalNAc, methyl *N*-acetyl-α-D-galactosaminide; SBA, soybean agglutinin; PBS buffer, phosphate-buffered saline; GS I-A_4 and GS I-B_4, *Griffonia simplicifolia* isolectins A_4 and B_4, respectively; TLC, thin-layer chromatography.

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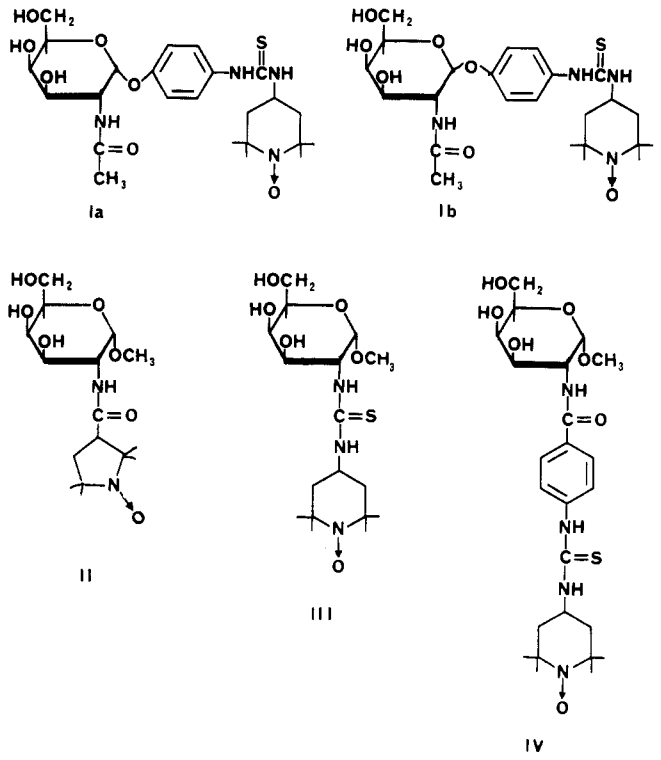

FIGURE 1: Structures of spin-labeled sugars examined in this study.

weights, respectively, as follows: LBL, 12.3 and 31 000 (Gould & Scheinberg, 1970); GS I-A_4 and I-B_4, 14.1 and 32 000 and 33 000, respectively (Hayes & Goldstein, 1974); *A. bracteata*, 13.6 and 32 000; *D. biflorus*, 11.2 and 27 500 (Etzler et al., 1981); SBA, 12.8 and 30 000 (Lotan et al., 1973).

Sugars. The structures of the spin-labeled sugars used in this study are shown in Figure 1. The synthesis of spin-labeled derivatives Ia [1-[4-[(*N*-acetyl-α-D-galactosaminyl)oxy]-phenyl]-3-(2,2,6,6-tetramethyl-1-oxypiperidin-4-yl)-2-thiourea], Ib [1,[4-[(*N*-acetyl-β-D-galactosaminyl)oxy]phenyl]-3-(2,2,6,6-tetramethyl-1-oxypiperidin-4-yl)-2-thiourea], and III [1-(methyl 2-deoxy-α-D-galactopyranosid-2-yl)-3-(2,2,6,6-tetramethyl-1-oxy-piperidin-4-yl)-2-thiourea] were prepared by the same procedure as described elsewhere (Plessas & Goldstein, 1981) except that, in this work, *N*-acetyl-D-galactosamine was substituted for galactose, as was methyl *N*-acetyl-α-D-galactosaminide (Neuberger & Wilson, 1971).

TLC was performed on precoated plates of silica gel G-60 (Brinkmann Instruments, Inc.); compounds were detected with a spray containing 5% each of ammonium molybdate, phosphoric acid, and sulfuric acid, followed by heating the plates for 10 min at 140 °C. Alternatively, plates were sprayed with 1% potassium permanganate in 10% sodium carbonate. Silica gel 60 (0.063–0.2-mm particle size, 70–230 mesh), used for column chromatography, was supplied by Brinkmann Instruments. The nitroxide moieties 3-carboxy-2,2,5,5-tetramethyl-1-oxypyrrolidine and 4-amino-2,2,6,6-tetramethyl-1-oxypiperidine were purchased from Eastman Kodak Co. Palladium hydroxide on carbon (the Pearlman catalyst) and thionyl chloride was obtained from Aldrich Chemical Co.

Synthesis of Spin-Label II [*Methyl 2-[[(2,2,5,5-Tetramethyl-1-oxypyrrolidin-3-yl)carbonyl]amino]-2-deoxy-α-D-galactopyranoside*]. (*a*) *3-(Chloroformyl)-2,2,5,5-tetramethyl-1-oxypyrrolidine.* A chilled mixture of 0.22 g (1.18 mmol) of 3-carboxy-2,2,5,5-tetramethyl-1-oxypyrrolidine in 4 mL of benzene and 0.12 mL of pyridine was treated with 0.11 ml of thionyl chloride added dropwise to the stirred solution. The reaction mixture was stirred at room temperature for 1 h, the precipitated salts were filtered and washed with dry benzene, and the filtrate was evaporated in vacuum at room temperature. Because the residual dark yellow crystalline acid chloride was readily hydrolyzed by moisture, it was prepared just prior to use.

(*b*) *Methyl 2-[[(2,2,5,5-Tetramethyl-1-oxypyrrolidin-3-yl)carbonyl]amino]-2-deoxy-α-D-galactopyranoside* (*II*). To a solution of 2-amino-2-deoxy-α-D-galactopyranoside (41.7 mg, 0.3 mmol) in 500 μL of 1 M sodium bicarbonate (pH 8.1) and 500 μL of methanol 0.12 g (0.59 mmol) of freshly prepared 3-(chloroformyl)-2,2,5,5-tetramethyl-1-oxypyrrolidine in 300 μL of acetone and 200 μL of methanol was added, and the reaction mixture was stirred at room temperature for 16 h. The red solution was evaporated to a syrup that was purified by silica gel column chromatography with 10:2 chloroform–methanol as the eluant. The product was crystallized from ether to give a hydroscopic yellow solid in 30% yield. Anal. Calcd for $C_{16}H_{29}N_2O_7 \cdot H_2O$: C, 50.64; H, 8.2; N, 7.38. Found: C, 50.08; H, 8.02; N, 7.10.

Synthesis of Spin-Label IV [*1-[4-[[(Methyl 2-deoxy-α-D-galactopyranosid-2-yl)amino]carbonyl]phenyl]-3-(2,2,6,6-tetramethyl-1-oxypiperidin-4-yl)-2-thiourea*]. A 1% solution (50 mg, 0.14 mmol) of methyl 2-deoxy-2-(*p*-nitrobenzamido)-α-D-galactopyranoside (Kaifu et al., 1985) in methanol was hydrogenated for 3 h in the presence of palladium hydroxide on carbon (50 mg) at room temperature and atmospheric pressure. The suspension was filtered and the filtrate evaporated to afford chromatographically pure methyl 2-deoxy-2-(*p*-aminobenzamido)-α-D-galactopyranoside (TLC in 2:1 chloroform–methanol, R_f 0.7).

A solution of methyl 2-deoxy-2-(*p*-aminobenzamido)-α-D-galactopyranoside (46 mg, 0.15 mmol) and 4-isothiocyanato-2,2,6,6-tetramethyl-1-oxypiperidine (Plessas & Goldstein, 1981; 49 mg, 0.23 mmol) in ethanol (20 mL) was boiled for 4 h under reflux. The red solution was evaporated to a syrup, which was purified by silica gel column chromatography with 10:3 chloroform–methanol as the eluant. The fractions having R_f 0.6 were combined and evaporated to a syrup in 65% yield, mp 185–187 °C, which solidified from ether to give a yellow hydroscopic solid. Anal. Calcd for $C_{24}H_{36}O_7N_3S$: C, 56.45; H, 7.1; N, 8.23; S, 6.28. Found: C, 55.92; H, 7.4; N, 8.67; S, 6.64.

N-Acetylglucosamine was purchased from Pfanstiehl (Waukegan, IL). Type A substance was a gift of Dr. R. Poretz, Rutgers University. Guaran was available from a previous study.

Precipitin Inhibition. Quantitative precipitation inhibition analysis was performed according to the procedure of Hammarstrom et al. (1977) and Murphy and Goldstein (1979). For *A. bracteata*, 33 μg of protein and 20 μg of type A substance were used in the equivalence mixture. Protein determinations were done by a micro Lowry procedure (Mage & Dray, 1965).

Electron Spin Resonance. ESR spectra were taken on a Varian E-4 spectrometer in quartz microcapillary tubes at 25 ± 2 °C (Berliner, 1978). All spectra were measured at X-band (9.5 GHz), 20-mW microwave power, 80- or 100-G field sweep, 0.5-G modulation amplitude, and 3395-G applied field. "High-gain" spectra were measured at a higher receiver gain and 2–8 times the modulation amplitude in order to estimate hyperfine splitting extreme. Specificity of sugar binding was tested by competitive displacement of excess haptenic or non-haptenic sugars. Lectin spin-label dissociation constants were determined as previously reported (Goldstein et al., 1985).

Table I: Summary of 2-*N*-(Oxypiperidinyl) or 2-*N*-(Oxypyrrolidinyl) Spin-Label–Lectin Interactions

	spin-label									
	II			III			IV			Me-α-GalNAc
lectin	K_d (mM)[a]	K_I (mM)	$2T_\parallel$ (G)	K_d (mM)	K_I (mM)	$2T_\parallel$ (G)	K_d (mM)	K_I (mM)	$2T_\parallel$ (G)	K_I (mM)
GS I-A_4	ND[b]	ND		*c*	0.81[d]	ND	0.22	0.13	48.5	0.1
GS I-B_4	ND	ND		*c*	1.6[d]	ND	*c*	ND	ND	ND
A. bracteata	1.0	1.7	66	2.6	1.6	69.5	0.10	0.17	50.5	0.15
LBL	3.0	ND	ND	ND	ND	ND	0.36	0.81	52.5	6.5
D. biflorus	3.0	ND	69.5	1.5	ND	ND	*c*	ND	ND	1.0
SBA	ND	ND	ND	ND	ND	ND	0.07	0.25	56.5	0.02

[a] From ESR spectra. [b] Not determined. [c] Weak binding, if any. [d] From Goldstein et al. (1985).

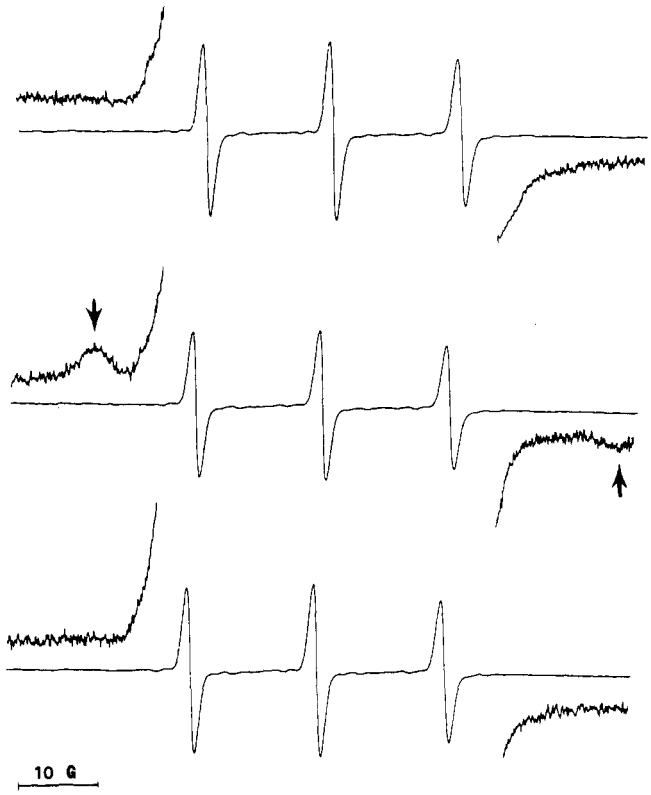

FIGURE 2: X-band ESR spectra of spin-label II and *A. bracteata* seed lectin: (upper spectrum) 180 μM spin-label II; (middle spectrum) 180 μM spin-label II and 222 μM *A. bracteata* seed lectin; (lower spectrum) after addition of 2.5 mM Me-α-GalNAc. Experimental conditions were as follows: 0.1 mM PBS buffer, pH 7.1; field set, 3395 G; scan range, 80 G; microwave frequency, 9.53 GHz; microwave power, 20 mW; gain, 2.5 × 10^4; modulation amplitude, 0.5 G; time constant, 0.25 s; scan time, 4 min. The high-gain portions of the spectra were recorded at twice the receiver gain and a modulation amplitude of 4 G, a time constant of 0.5 G, and a scan time of 16 min.

The hyperfine extreme $2T_\parallel$ are estimated to within ±0.3 G. The K_d values are accurate to within 15%.

RESULTS

Binding of 2-N-(Oxypiperidinyl) or 2-N-(Oxypyrrolidinyl) Spin-Labels. Spin-labeled sugars II–IV form a series of derivatives with 2-*N*-(oxypiperidinyl) or 2-*N*-(oxypyrrolidinyl) substituents varying in length and polarity of the linker moiety between the glycoside and nitroxide group. Dissociation constants were estimated from the difference in free line intensity of the spin-label in the absence and presence of lectin, which also agree well with hapten inhibition data. In all cases, the spin-label was displaced completely by methyl *N*-acetyl-α-D-galactosaminide (25–30 mM) but not by *N*-acetyl-D-glucosamine. GS I-B_4, which is specific for terminal, nonreducing α-D-galactosyl groups, did not bind (or bound very poorly) with any of these ligands. Spin-labels II and III, which have the nitroxyl group close to the 2-amino group of galactosamine, bound weakly, if at all, to the lectins tested. In contrast, spin-label IV was bound very well and, with the exception of the *D. biflorus* lectin, proved to be a very good inhibitor of the precipitin reaction.

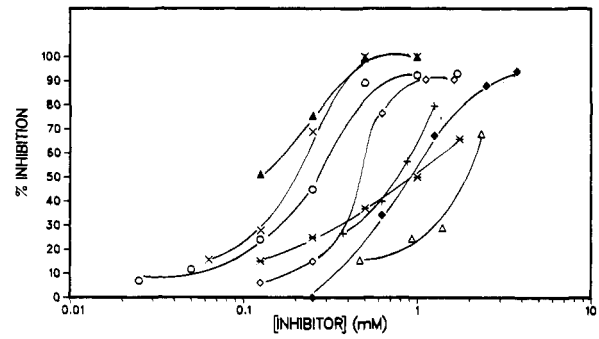

FIGURE 3: Precipitin inhibition by spin-labeled galactosamines. Inhibition of quantitative precipitation was performed by adding increasing concentrations of inhibitor to mixtures of lectin and glycoconjugate in a final volume of 200 μL. The following mixtures were used: 33 μg of *A. bracteata* lectin and 20 μg of human blood group A substance; 30 μg of GS I-A_4 and 10 μg of guaran; 47 μg of LBL and 15 μg of human blood group A substance; 38 μg of soybean agglutinin and 10 μg of guaran. GS I-A_4 and *A. bracteata* lectin mixtures were incubated at 25 °C for 48 h; LBL and soybean agglutinin were incubated at 4 °C for 5 days. (△) *A. bracteata* lectin plus spin-label II; (+) *A. bracteata* lectin plus spin-label III; (×) *A. bracteata* lectin plus spin-label IV; (◇) *A. bracteata* lectin plus spin-label Ib; (▲) GS I-A_4 plus spin-label IV; (*) GS I-A_4 plus spin-label Ib; (◆) LBL plus spin-label IV; (○) SBA plus spin-label IV.

The *D. biflorus* lectin bound all spin-labels too weakly (K_d > 1.5 mM) to obtain reliable K_d or K_I values. *A. bracteata* lectin showed measurable affinity for II (K_d = 1.0 mM) with a high degree of immobilization of the nitroxide ring ($2T_\parallel$ = 66 G) (Figure 2). Spin-label III was strongly immobilized upon binding to this lectin ($2T_\parallel$ = 69.5 G), albeit with a very weak K_d = 2.6 mM. Spin-label IV, on the other hand, bound strongly to *A. bracteata* lectin (K_d = 0.1 mM) but with very little immobilization ($2T_\parallel$ = 50.5 G) of the nitroxide ring (see Table I). Hapten inhibition and ESR results indicated that spin-label IV had a similar affinity for *A. bracteata* lectin, soybean agglutinin, and the *G. simplicifolia* I-A_4 isolectin as did Me-α-GalNAc (Figure 3, Table I). Immobilization of the nitroxyl moiety by these lectins ranged from weak ($2T_\parallel$ = 48.5 G for GS I-A_4) to moderate ($2T_\parallel$ = 56.5 G) for soybean agglutinin. The affinity of spin-label IV for LBL was 18-fold greater (K_d = 0.36 mM) than for Me-α-GalNAc (K_d = 6.5 mM), while the hyperfine splitting ($2T_\parallel$ = 52.5 G) was again indicative of a weak to moderate immobilization of the oxypiperidine ring by the protein.

Binding of N-Acetyl-D-galactosamine Glycosides Spin-Labeled in the Aglycon. The interaction of GS I-A_4 and *A.*

Table II: Summary of Binding of Glycosidic Spin-Labels

	spin-label					
	Ia			Ib		
lectin	K_d (mM)	K_I (mM)	$2T_\parallel$ (G)	K_d (mM)	K_I (mM)	$2T_\parallel$ (G)
GS I-A	*b*	2.0	ND[a]	0.27	0.63	48.8
A. bracteata	*b*	2.0	ND	0.14	0.28	49.2
LBL	*b*	ND	ND	3.0	ND	
D. biflorus	3.0	ND	ND	2.0	ND	

[a] Not determined. [b] Weak binding, if any.

bracteata lectins with α- and β-anomers of glycosidically linked spin-labels Ia and Ib, respectively, is summarized in Table II. Surprisingly, the α-anomer did not bind to either lectin; however, the β-anomer (Ib) bound to GS I-A_4 with an affinity similar to that seen with *p*-nitrophenyl-β-D-GalNAc (Murphy & Goldstein, 1979). It was clear from the ESR spectra for spin-label Ib binding to GS I-A_4 (not shown) that the binding was specific, since it could be totally obliterated by addition of 25 mM GalNAc. It appears that in the α-anomeric position the oxypiperidinyl moiety hinders binding whereas it is tolerated in the β-anomer. Additionally, the phenyl group spacer in the β-anomeric configuration is known to enhance binding to GS I-A_4 over the α-anomer by virtue of hydrophobic interactions with the nonpolar region adjacent to the carbohydrate binding site (Murphy & Goldstein, 1979). LBL and *D. biflorus* agglutinin bound these ligands very weakly (Table II).

Discussion

A notable observation of this study was that of all the spin-labels studied spin-label IV, containing the hydrophobic 2-*N*-phenyl group, showed the strongest interaction with all lectins tested except that from *D. biflorus*. The extent of the interaction between the ligand and the protein was inferred from the hyperfine extrema splittings, which are related to the rotational freedom of the nitroxide ring (Berliner, 1978). Values for $2T_\parallel$ are also listed in Table I.

A. bracteata lectin provided an interesting example of interactions with the spin-label series II–IV. Spin-label II showed weak binding (1.0 mM) yet a high degree of immobilization of the label ($2T_\parallel$ = 66 G) (see Figure 2). On the other hand, spin-label III, with the nitroxide group at approximately 3 Å further from the glycoside, displayed lower affinity than II, yet again was strongly immobilized. Spin-label IV had a much higher affinity than II and III but very little immobilization of the nitroxide ring, $2T_\parallel$ = 50.5 G (Table I). This suggests that the oxypiperidinyl and oxypyrrolidinyl groups were poorly accommodated by the *A. bracteata* carbohydrate binding site yet were rotationally hindered upon binding. The phenyl group in IV, however, appeared to facilitate binding of the spin-labeled analogues, perhaps by strong hydrophobic interactions near the combining site and also by placing the bulky oxypiperidinyl group at some distance from the protein surface, as demonstrated by its decreased immobilization. Hapten inhibition results with methyl *N*-(*p*-nitrobenzamido)-α-D-galactosaminide showed this ligand to also have the same K_I as Me-α-GalNAc (data not shown).

In the case of the lima bean and *D. biflorus* lectins, the binding of spin-label IV is consistent with precipitin inhibition results using methyl *N*-*p*-nitrobenzamido)-α-D-galactosaminide (Galbraith & Goldstein, 1972; Hammarstrom et al., 1977). Enhanced binding was observed with both ligands to LBL. The somewhat weak immobilization of the ligand again suggested that the nitroxide was at some distance from the protein. No binding was seen with *D. biflorus* lectin, consistent with the observed restricted binding site of this lectin (Hammarstrom et al., 1977).

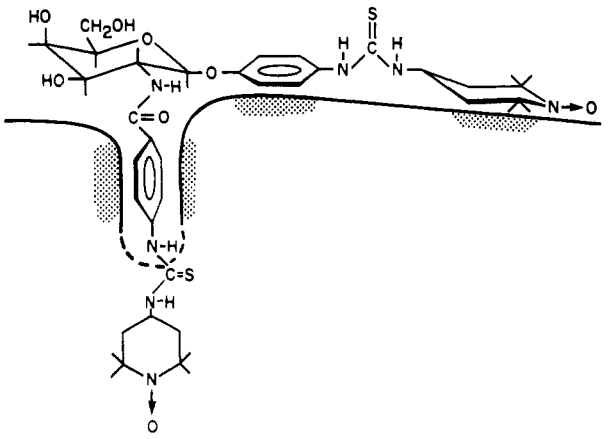

FIGURE 4: Diagram of the composite binding of spin-labels Ib and IV to *A. bracteata* and GS I-A_4 lectins illustrating the hydrophobic interactions between the phenyl groups and nonpolar regions (hatched areas) of the lectins.

As with *A. bracteata*, GS I-A_4 and SBA had similar affinities for spin-label IV and Me-α-GalNAc (Table I). In ESR experiments, soybean agglutinin showed a strong interaction with the oxypiperidinyl group, perhaps due to a more extended hydrophobic binding site than in the former two lectins. In ESR experiments, soybean agglutinin showed a strong interaction with the oxypiperidinyl group, perhaps due to a more extended hydrophobic binding site than in the former two lectins. This is supported by the results of DeBoeck et al. (1984), who showed that the hydrophobic dansyl group made a large entropic contribution to the enhanced binding of *N*-dansylgalactosamine to SBA.

Among the lectins examined, it is possible to discern variations in the nature of the binding sites ranging from one that is restricted to recognition only of the carbohydrate moiety (*D. biflorus*) to those with an extended binding site that includes hydrophobic regions.[2] For those lectins where the observation that high affinity did not necessarily correlate with strong immobilization, we may speculate that the binding sites are not greatly extended; that is, the rapid rotational rate of the nitroxide group was consistent with a lack of interaction with the protein surface.

The results obtained with spin-labels Ia and Ib were interesting in light of similar studies with 1-[4-(β-galactopyranosyloxy)phenyl]-3-(2,2,6,6-tetramethyl-1-oxypiperidin-4-yl)-2-thiourea and 2,2,6,6-tetramethyl-1-oxypiperidin-4-yl α-D-galactopyranoside (Goldstein et al., 1985). The *p*-aminophenyl α-galactoside bound very weakly to GS I-A_4, whereas the oxypiperidinyl α-galactoside bound with relatively greater affinity. In contrast here, Ia bound very poorly to all lectins tested (Table II) while Ib bound reasonably well to GS I-A_4 and *A. bracteata* (see Table II). It appears that the α-anomer positions the oxypiperidinyl moiety such that binding is hindered while the β-anomer does not.

The interaction of hydrophobic aglycon moieties with GS I-A_4 can be ranked as follows: *p*-nitrophenyl-α-GalNAc > *p*-nitrophenyl-β-GalNAc > *p*-aminophenyloxypiperidinyl-β-GalNAc ≫ *p*-aminophenyl-α-GalNAc. Results with *A. bracteata* were similar: *p*-nitrophenyl-α-GalNAc > *p*-nitrophenyl-β-GalNAc > *p*NH_2-aminophenyloxypiperidinyl-β-

[2] Although not addressed specifically in this study, differences in the polarity of the microenvironment have also been shown to affect the hyperfine splitting by a few gauss (Griffith et al., 1974).

GalNAc ≫ p-aminophenyloxypiperidinyl-α-GalNAc (unpublished results). This suggests that the bulky nitroxyl moiety interfered with binding when at some distance from the immediate sugar binding site. Figure 4 depicts a composite binding model for the spin-label analogues examined in this study.

All of the *N*-acetyl-D-galactosamine binding lectins included in this study belong to the family *Leguminoseae*; *A. bracteata* and *D. biflorus* are also classified under the same subfamily *Papilionoideae*. As the primary sequences of legume lectins have become available, extensive homologies among them have become apparent. Thus, not only the determination of their ligand binding similarities but the differences among them may eventually provide insights into the physiological role of these proteins in vivo.

Registry No. Ia, 102650-31-7; Ib, 102650-32-8; II, 102650-33-9; III, 77895-27-3; IV, 102650-34-0; Me-α-GalNAc, 6082-22-0; 3-(chloroformyl)-2,2,5,5-tetramethyl-1-oxypyrrolidine, 61593-19-9; 3-carboxy-2,2,5,5-tetramethyl-1-oxypyrrolidine, 2154-68-9; 2-amino-2-deoxy-α-D-galactopyranoside, 14196-84-0; methyl 2-deoxy-2-(*p*-nitrobenzamido)-α-D-galactopyranoside, 20581-53-7; methyl 2-deoxy-2-(*p*-aminobenzamido)-α-D-galactopyranoside, 20581-55-9; 4-isothiocyanato-2,2,6,6-tetramethyl-1-oxypiperidine, 36410-81-8.

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Kinetic Comparison of Ricin Immunotoxins: Biricin Conjugate Has Potentiated Cytotoxicity

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Received October 15, 1985; Revised Manuscript Received March 26, 1986

ABSTRACT: The plant toxin ricin was chemically coupled to an anti-Thy-1.1 antibody, and the resultant conjugates were fractionated by gel filtration. The cytotoxicity of the conjugate possessing two ricin molecules per immunoglobulin, yielding a first-order inactivation rate of protein synthesis of −0.4 log/h at 200 ng/mL, was well above that expected just from the increase in ricin per unit mass of conjugate, when compared to a conjugate possessing only one ricin per immunoglobulin. On a conjugate molar scale the biricin immunotoxin was determined to be 8 times more potent than the monoricin conjugate; thus, relative to the number of ricin molecules, the coupling of a second ricin to the immunoglobulin quadrupled the observed potency. The concentration of immunotoxin and the resultant inactivation rates of protein synthesis were found to be related through a power function. Additionally, the inactivation kinetics of these conjugates were found to be similar to those of native ricin.

The purpose in synthesizing an immunotoxin is to combine the specificity of an antibody with the cytotoxic features of a natural toxin [for review see Neville (1985)]. Clinical application of an immunotoxin would involve the selection of an antibody directed against a pathological or undesirable group of cells; however, natural toxins lack the desired specificity and will bind to and kill most desirable nontarget cells. The plant toxin ricin, a lectin with specificity for galactose-ter-

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