Binding and Precipitating Activities of *Lotus tetragonolobus* Isolectins with L-Fucosyl Oligosaccharides. Formation of Unique Homogeneous Cross-Linked Lattices Observed by Electron Microscopy†

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ABSTRACT: We have recently observed that certain asparagine-linked oligosaccharides are multivalent and capable of binding and precipitating with the D-mannose-specific lectin concanavalin A [cf. Bhattacharyya, L., & Brewer, C. F. (1989) *Eur. J. Biochem. 178*, 721–726] and with a variety of D-galactose-specific lectins [Bhattacharyya, L., Haraldsson, M., & Brewer, C. F. (1988) *Biochemistry 27*, 1034–1041]. In the present study, we have examined the binding and precipitating activities of a variety of mono- and biantennary L-fucosyl oligosaccharides with three L-fucose-specific isolectins from *Lotus tetragonolobus*, LTL-A, LTL-B, and LTL-C. The results show that certain difucosyl biantennary oligosaccharides are capable of cross-linking and precipitating with tetrameric isolectins, LTL-A and LTL-C, but not with dimeric isolectin, LTL-B. Quantitative precipitation analyses show that biantennary oligosaccharides containing the Lewis^x antigen (or type 2 chain of Lewis^a), Galβ(1–4)[Fucα(1–3)]GlcNAc, at the nonreducing terminus of each arm are bivalent ligands. However, a biantennary oligosaccharide containing a Lewis^x determinant on one arm and a type 2 chain of blood group H(0) determinant, Fucα(1–2)Galβ(1–4)GlcNAc, on the other arm and a monoantennary oligosaccharide containing two fucose residues (analogue of the Lewis^y antigen) bind but do not precipitate with the isolectins, indicating that the positions and linkage of fucose residues are critical for cross-linking. The presence or absence of the galactose residues in the Le^x antigenic determinant(s) of the oligosaccharides does not affect the affinities of the carbohydrates for the isolectins but has a strong influence on the temperature sensitivity and kinetics of the precipitation reactions with LTL-A and LTL-C. On the other hand, the presence of the α(1–3) fucose residues in the oligosaccharides with terminal β(1–4) galactose residues eliminates binding of the carbohydrates to certain galactose-specific lectins. The precipitates formed between the oligosaccharides and LTL-A and LTL-C have also been examined by electron microscopy. The precipitates of LTL-A show a distinct lattice pattern for each oligosaccharide, indicating the presence of long-range order and well-defined geometry in each cross-linked complex. However, the precipitates of LTL-C show no pattern. The precipitates of LTL-A formed in the presence of a mixture of two oligosaccharides show patterns characteristic of one or the other oligosaccharide depending on the relative concentrations of the carbohydrates. Similar findings were also obtained with the precipitates of binary mixtures of LTL-A and LTL-C with one oligosaccharide. These studies thus demonstrate the formation of distinct homogeneous cross-linked lattices between LTL-A and three bivalent oligosaccharides, as well as different stabilities for the cross-linked lattices of LTL-A and LTL-C with a given oligosaccharide. The results are discussed in terms of the structure–function properties of multivalent oligosaccharides and lectins.

Oligosaccharides containing fucose[1] residues are found in glycoproteins (Santer et al., 1983; Pierce-Cretel et al., 1984; Kornfeld & Kornfeld, 1985; Rao & Biswas, 1985) as well as in glycolipids (Hakomori & Kannagi, 1983; Hakomori, 1984; Marcus, 1984). Fucose residues are also essential components of many antigenic determinants, including the Le^a, Le^b, Le^x, poly-Le^x, Le^y, and poly-Le^y antigens[2] (Hakomori & Kobata, 1974; Watkins, 1980; Rao & Biswas, 1985). The expression of fucosyl oligosaccharides on the surface of cells, particularly the Le^x antigenic determinants (or type 2 chain of Le^a), Galβ(1–4)[Fucα(1–3)]GlcNAc, has been found to be developmentally regulated [cf. Sharon (1983)] and altered as a result of differentiation (Muramatsu et al., 1982) and oncogenic transformation (Hakomori & Kannagi, 1983; Hakomori, 1984). Increased expression of Le^x antigens has been observed

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[1] Fucose is in the L configuration; all other sugars are in the D configuration.

[2] Abbreviations: Le^a and Le^b, subtypes a and b of the blood group Lewis antigens (Watkins, 1980); Le^x and Le^y, Lewis^x and Lewis^y antigens, these antigens were previously designated X and Y antigens (Hakomori & Kobata, 1974), but the present nomenclature follows from the fact that they are positional isomers of blood group Lewis antigens [however, it is known now that these antigens are different from blood group Lewis antigens (Hakomori, 1984)]; ConA, concanavalin A, the glucose/mannose-specific specific lectin from the seeds of *Canavalia ensiformis*; LTL-A, LTL-B, and LTL-C, the isolectins A, B, and C, respectively, from *Lotus tetragonolobus*; EIL, lectin from the seeds of *Erythrina indica*; RCA-I, Agglutinin I from the seeds of *Ricinus communis*.

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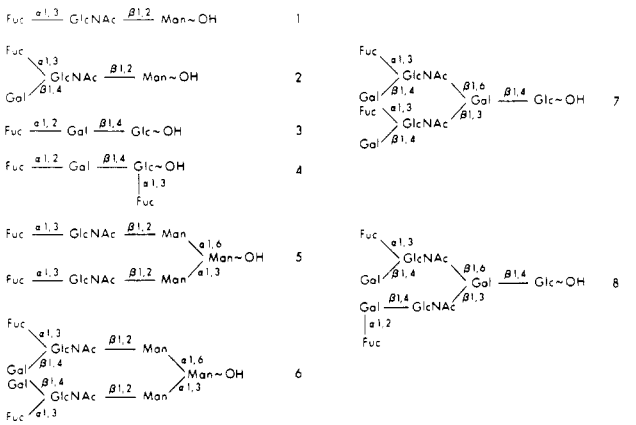

FIGURE 1: Structures of fucosyl mono- and biantennary oligosaccharides. Fuc, Gal, Man, and GlcNAc represent fucose, galactose, mannose, and *N*-acetylglucosamine residues, respectively.

in most common human cancers, particularly in adenocarcinoma (Hakomori et al., 1984) and neuroblastoma (Santer et al., 1983). Oligosaccharides containing fucose have also been implicated in recognition involving the fertilization of eggs by sperm (Glabe et al., 1982). Thus, evidence suggests that fucosyl oligosaccharides are important cell surface recognition determinants.

Although considerable work has been done to detect fucosyl oligosaccharides on the surface of cells and determine their structures, relatively little is known about their molecular binding properties except for limited studies on their interactions with glycosidases and glycosyltransferases (Schachter & Roseman, 1980; Snider, 1984), monoclonal antibodies (Hakomori & Kannagi, 1983; Hakomori, 1984; Marcus, 1984), and lectins, which are multivalent saccharide binding proteins [cf. Goldstein and Poretz (1986)].

We have been studying the interactions of asparagine-linked (N-linked) oligosaccharides with lectins in order to gain insight into the molecular binding properties of the carbohydrates and proteins as receptors. We have shown that certain N-linked oligosaccharides are multivalent and capable of cross-linking and precipitating with lectins (Bhattacharyya et al., 1987a,b, 1988a, 1989a; Bhattacharyya & Brewer, 1989). Our recent studies have indicated that the specificity of lectin–carbohydrate interactions is much greater in cross-linked complexes than in soluble complexes. For example, quantitative precipitation analyses of cross-linking of concanavalin A (ConA) with binary mixtures of a series of oligomannose and bisected hybrid-type glycopeptides, which have closely related structures, indicate that each glycopeptide forms a homogeneous cross-linked lattice with the lectin (Khan et al., 1988; Bhattacharyya et al., 1988b). Heterogeneous complexes containing two different glycopeptides bound to ConA fail to precipitate and exist only as soluble complexes. These findings suggest that carbohydrates that have closely related structures each form unique cross-linked lattices with a given lectin.

In the present study, we have investigated the interactions of a variety of mono- and biantennary fucosyl oligosaccharides (Figure 1) with the fucose-specific isolectins A, B, and C from *Lotus tetragonolobus* (LTL-A, LTL-B, and LTL-C, respectively). The results show that biantennary oligosaccharides **5**–**7** are capable of precipitating as bivalent ligands with tetrameric isolectins LTL-A and LTL-C, but not with dimeric isolectin LTL-B. In order to test the hypothesis that structurally related oligosaccharides each produce unique cross-linked lattices with a lectin, we have examined the precipitates of LTL-A and LTL-C with oligosaccharides **5**–**7** by electron microscopy. The results show that the precipitates of LTL-A with each oligosaccharide possess highly organized, unique lattices. Competition experiments with binary mixtures of the oligosaccharides also indicate that each oligosaccharide forms a lattice that excludes the other oligosaccharide. The electron micrographs also demonstrate differences in the stabilities of the cross-linking complexes of LTL-A and LTL-C with the oligosaccharides.

Materials and Methods

Seeds of *Lotus tetragonolobus* (syn. *Tetragonolobus purpureas*) were purchased from Schumacher & Co., MA. The native lectin mixture was purified from the crude extract [prepared according to Yariv et al. (1967)] by affinity chromatography as described previously (Allen & Johnson, 1977). The isolectins, LTL-A, LTL-B and LTL-C, were separated by DEAE-cellulose chromatography (Kalb, 1968). The concentrations of LTL-A, LTL-B, and LTL-C were measured spectrophotometrically by using $A^{1\%,1cm}$ values of 17.4, 20.9, and 17.8, respectively (Kalb, 1968), and expressed in terms of monomers. Lectin from the seeds of *Erythrina indica* (EIL) was purified as described (Bhattacharyya et al., 1981). Agglutinin I from *Ricinus communis* (RCA-I) was purchased from Sigma Chemical Co., St. Louis, MO. The syntheses of oligosaccharides **1**, **2**, **5**, and **6** (Figure 1) were described previously (Lonn, 1985a,b). Oligosaccharides **4**, **7**, and **8** (Figure 1) were products of BioCarb Chemicals, Sweden. Oligosaccharide **3** and fucose were obtained from Sigma Chemical Co., St. Louis, MO. The concentrations of sugars were measured by the phenol–sulfuric acid method (Dubois et al., 1956) using appropriate mixtures of fucose, mannose, and galactose as standards.

Hemagglutination Inhibition Assays. These were done at room temperature by the 2-fold serial dilution technique (Osawa & Matsumoto, 1972) in 10 mM sodium phosphate buffer, pH 7.2, containing 0.15 M NaCl, using a 3% (v/v) suspension of human blood group O erythrocytes.

Quantitative Precipitation Assays. The assays were performed in a final volume of 0.1 mL by using 0.1 M Tris-HCl buffer, pH 7.2, containing 0.9 M KCl, 1 mM $MnCl_2$, and 1 mM $CaCl_2$ as described previously (Bhattacharyya et al., 1987a,b). To examine the effects of salt and bivalent metal ions, precipitation reactions were also carried out in 10 mM sodium phosphate buffer, pH 7.2, containing 0.15 M NaCl.

Kinetics of Precipitation. These were done by following the time course of development of turbidity at 420 nm in Uvonic Type 17Q cells (path length = 1 cm) in a Gilford 260 spectrophotometer coupled with a Cole-Palmer Model 8373-10 recorder. The temperature was kept constant at 5.0 ± 0.1 °C by an Endocal RTE-9B temperature control bath. The buffer was 0.1 M Tris-HCl, pH 7.2, containing 0.9 M KCl, 1 mM $MnCl_2$, and 1 mM $CaCl_2$. The solutions were gently stirred and the absorbances were monitored continuously until they remain essentially constant for 15 min for oligosaccharide **5** and 4 h for oligosaccharides **6** and **7**.

Electron Microscopy. Precipitates were negatively stained by placing the samples on 300 mesh carbon-coated Parlodion grids that had been freshly glow discharged, touched to filter paper, floated on a drop of 1% phosphotungstic acid, pH 7.0, and blotted immediately. Samples were observed at 80 kV in a JEOL 1200EX electron microscope. For freeze fracture studies, samples were placed in a gold double-replica device, frozen in liquid Freon, and fractured in a Balzers BAF301 freeze-fracture unit at −115 °C. The fracture face was shadowed at a 45° angle with platinum and stabilized with carbon. Samples were observed as described above.

Table I: Minimum Concentrations of Sugars Required for Inhibition of Hemagglutination by Lectins

	min concn (mM) of carbohydrates required to completely inhibit hemagglutination by[a]				
oligosacch	LTL-A	LTL-B	LTL-C	EIL	RCA-I
fucose	1.6	3.1	3.1		
lactose				3.1	3.1
1	1.5	1.6	3.0		
2	1.5	1.2	3.0	>12.0	>12.0
3	1.6			3.3	>13.0
4	1.5	2.9	3.0		
5	1.2		2.3		
6	1.8			>10.0	
7	7.9				
8	8.7				

[a] In each case, the lectin concentration was adjusted to a hemagglutination titer of 8 (Osawa & Matsumoto, 1972).

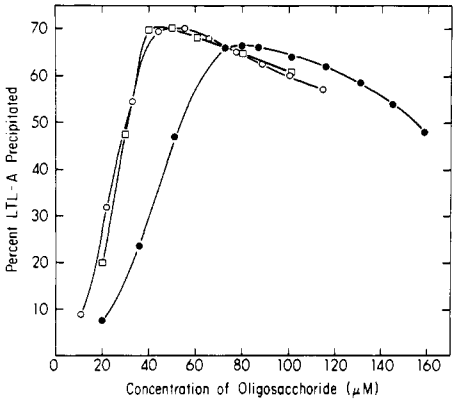

FIGURE 2: Precipitation profiles of LTL-A in the presence of oligosaccharides **5** (○), **6** (□), and **7** (●) at 4 °C. See Table II for protein concentrations.

RESULTS

Inhibition Studies. Table I summarizes the results of inhibition by fucose and the fucosyl oligosaccharides (Figure 1) of hemagglutination by LTL-A, LTL-B, and LTL-C. Table I also shows the results of inhibition by lactose and oligosaccharides **2**, **3**, and **6** of hemagglutination by the galactose-specific lectins EIL and RCA-I. Fucose appears to have a slightly higher affinity for LTL-A compared to LTL-B and LTL-C. Oligosaccharides **1**–**6** have essentially the same affinities for LTL-A as fucose, and oligosaccharides **7** and **8** have approximately 5-fold weaker affinities. The affinities of **1**, **2**, and **4** for LTL-B and LTL-C are approximately the same as the affinities of fucose for the respective isolectins. A similar result was also found with oligosaccharide **5** for LTL-C. The affinities of oligosaccharide **5** for LTL-B and that of **7** and **8** for LTL-B and LTL-C could not be measured due to the lack of materials.

Oligosaccharide **3** binds EIL as strongly as lactose but does not inhibit hemagglutination by RCA-I. Oligosaccharides **2** and **6**, which have galactose residues at the nonreducing termini, did not inhibit hemagglutination by EIL at 12 and 10 mM, respectively. Also, oligosaccharide **2** did not inhibit hemagglutination by RCA-I at 12 mM.

Quantitative Precipitation Studies. Figures 2 and 3 show quantitative precipitation profiles for LTL-A and LTL-C, respectively, in the presence of oligosaccharides **5**–**7** at 4 °C. The concentration of oligosaccharide at the equivalence point (point of maximum precipitation) (Kabat, 1976) of each precipitation profile and the concentration of protein monomer are shown in Table II. The ratio of the concentrations gives the stoichiometry of the precipitation reaction (Kabat, 1976),

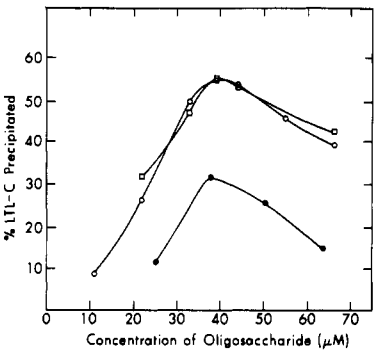

FIGURE 3: Precipitation profiles of LTL-C in the presence of oligosaccharide **5** in Tris-HCl (○) and sodium phosphate (□) buffers and **6** (●). See Table II for protein concentrations.

Table II: Stoichiometries of Precipitation Reactions of LTL-A and LTL-C with Fucosyl Complex Type Oligosaccharides at 4 °C[a]

oligosacch	lectin concn (μM)	concn oligosacch at equivalence pt (μM)	stoichiometry precipitation reactn	% lectin precipitated at equivalence pt
		LTL-A		
5	90	48	1:1.9	69
	90[b]	50	1:1.8	70
	86[b,c]	47	1:1.8	65
6	84	44	1:1.9	70
	83[c]	44	1:1.9	66
7	102	80	1:1.3	67
		LTL-C		
5	76	39	1:1.9	55
	76[c]	39	1:1.9	56
6	70	38	1:1.8	33

[a] Unless stated otherwise the data are from assays in 0.1 M Tris-HCl buffer, pH 7.2, containing 0.9 M KCl, 1 mM $MnCl_2$, and 1 mM $CaCl_2$. [b] Data at 22 °C. [c] Data in 10 mM sodium phosphate buffer, pH 7.2, containing 0.15 M NaCl.

which is also included in Table II. In each case, the precipitates dissolve upon addition of 0.1 M fucose and are prevented from forming in the presence of the monosaccharide.

At the protein concentrations used, oligosaccharide **5** precipitates with LTL-A at both 4 and 22 °C (profile at 22 °C not shown), but oligosaccharides **6** and **7** precipitate with the protein only at 4 °C (Figure 2). At either temperature, oligosaccharide **5** precipitates about 70% of LTL-A at the equivalence point, as do **6** and **7** at 4 °C. Oligosaccharide **8**, which is an isomer of **7**, oligosaccharide **4**, which has two fucose residues on the same chain, and monoantennary oligosaccharides **1**–**3** failed to precipitate with LTL-A at either 22 or 4 °C even at a lectin concentration as high as 150 μM.

Oligosaccharides **5** and **6** did not precipitate with LTL-C at 22 °C, but did so only at 4 °C (Figure 3). The results show that the precipitation of LTL-C at 4 °C is somewhat weaker with **5** and significantly weaker with **6** compared to the precipitation of LTL-A with the respective oligosaccharides (Table II). The complete precipitation profile for LTL-C with oligosaccharide **7** could not be done due to the lack of materials. However, preliminary experiment shows that oligosaccharide **7** also precipitates with LTL-C. As with LTL-A oligosaccharides **1**–**4** and **8** did not precipitate with LTL-C.

Table II indicates that in each case the stoichiometry of reaction of the oligosaccharides with the isolectins is approximately 1:2. Precipitation of LTL-A with oligosaccharide **7** shows 1:1.3 stoichiometry, which deviates from 1:2 stoichiometry due to weak binding. Since both LTL-A and LTL-C contain one carbohydrate binding site per monomer (Kalb, 1968), the results indicate that the oligosaccharides are

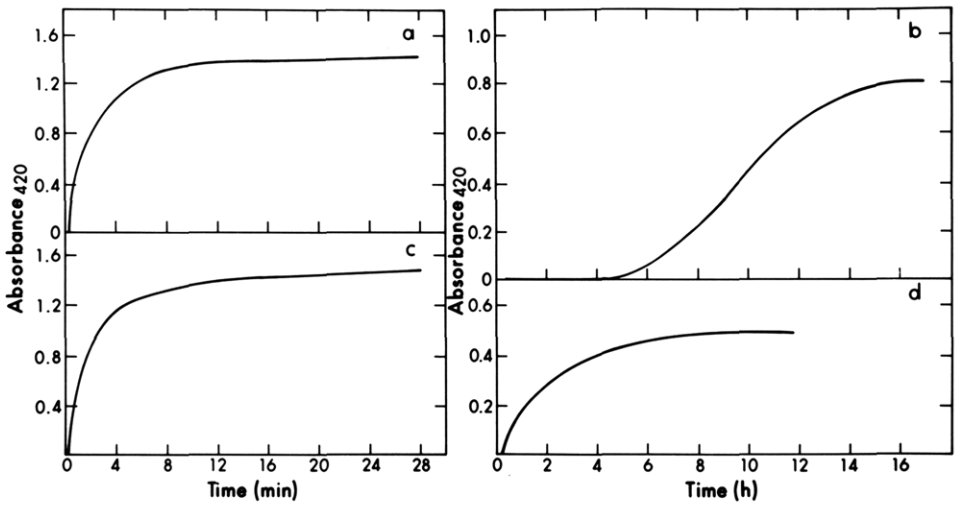

FIGURE 4: Profiles for the kinetics of appearance of precipitates of LTL-A (a, b) and LTL-C (c, d) in the presence of the oligosaccharides **5** (a, c) and **6** (b, d) at 5 °C. See Table III for protein and oligosaccharide concentrations.

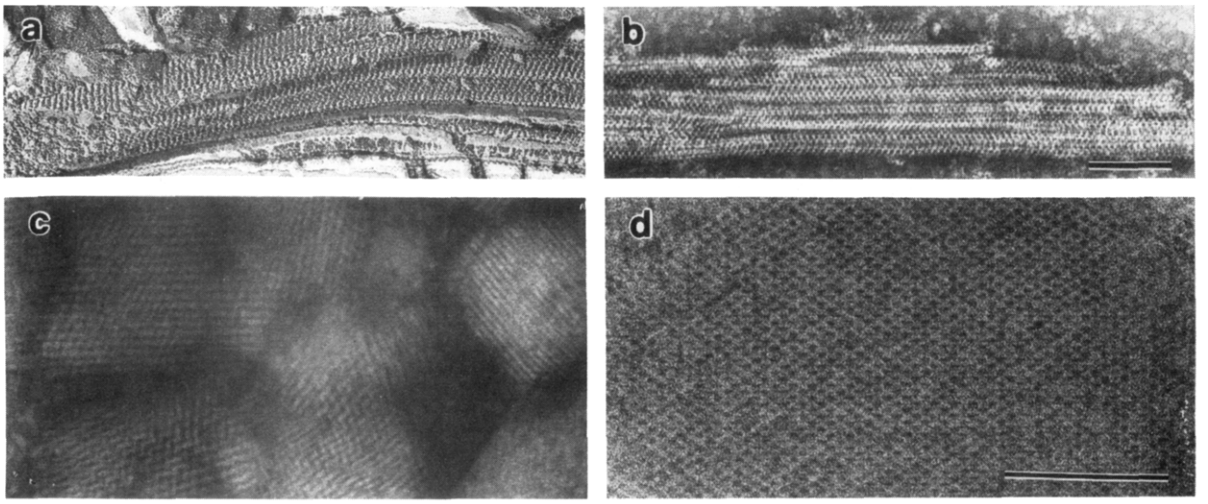

FIGURE 5: Electron micrographs of the precipitates of LTL-A (100 μM) with fucosyl biantennary oligosaccharides. (a) Freeze-fracture pattern with **5** (60 μM); (b) negative-stain pattern with **5** (60 μM); (c) negative-stain pattern with **6** (60 μM); (d) negative-stain pattern with **7** (90 μM). The bars in b and d represent 0.1 μm. Magnification in a, b, and c is the same.

divalent for binding to the isolectins.

Experiments in either 0.1 M Tris-HCl buffer, pH 7.2, containing 0.9 M KCl, 1 mM $MnCl_2$, and 1 mM $CaCl_2$ or 10 mM sodium phosphate buffer containing 0.15 M NaCl show that the precipitation profiles for LTL-A with oligosaccharide **5** are very similar, and the extent of precipitation at the equivalence points of respective precipitation profiles are approximately the same. Similar results are also obtained for precipitation of LTL-A by **6** and precipitation of LTL-C by both oligosaccharides. The results indicate that the precipitation reactions are insensitive to buffer ions, salt concentrations, and the presence or absence of Mn^{2+} and Ca^{2+} ions in the buffer.

None of the oligosaccharides precipitated with LTL-B at 4 or 22 °C.

The formation of precipitates of LTL-A and LTL-C with oligosaccharides **5**–**7** did not occur in the presence of 0.1 M fucose, and the precipitates were dissolved upon addition of the monosaccharide, thereby demonstrating the specificity of the interactions underlying the cross-linking reactions.

Kinetics of Precipitation. Figure 4 shows the time course of precipitation of LTL-A and LTL-C in the presence of oligosaccharides **5** and **6** at 5 °C. The protein and oligosaccharide concentrations are given in Table III, as are the half-time ($t_{1/2}$) and the initial delay time for the appearance of precipitates in each case. Kinetic data for the precipitation of **7** with LTL-A are also included in Table III. In each case, the protein concentration is essentially the same as that used in the quantitative precipitation analysis, and the concentration of oligosaccharide corresponds to the concentration at the equivalence point of the precipitation profile. The kinetic data are generated in the presence of equivalent amounts of oligosaccharide and lectin, and thus the results are directly comparable for different mixtures.

The results in Table III show that oligosaccharide **5** precipitates LTL-A significantly faster than **6** and **7** at 5 °C. Furthermore, the onset of precipitation of LTL-A with **5** is much more rapid compared to the precipitation of the same protein with **6** and **7**. Oligosaccharide **5** also precipitates LTL-C faster than **6** at 5 °C (Figure 4), with longer delay times for the onset of precipitation for **6** compared to **5** (Table III). However, the differences in the kinetic parameters between **5** and **6** are less dramatic for the precipitation of LTL-C than for LTL-A.

Electron Microscopy of Precipitates with Individual Oligosaccharides. Figure 5 shows the electron micrographs of the precipitates of LTL-A in the presence of oligosaccharides **5**–**7**. Figure 5a shows the freeze-fracture pattern of the precipitates of LTL-A with **5**. Figure 5b shows the negative-stain pattern of the same precipitates. The latter pattern has been

Table III: Kinetics of Precipitation of LTL-A and LTL-C with Oligosaccharides **5**, **6**, and **7** at 5 °C

oligosacch	protein concn (μM)	oligosacch concn (μM)	$t_{1/2}$[a]	delay time
		LTL-A		
5	90	48	1.8 min	0.1 min
6	84	44	10 h	5 h
7	102	80	21 h	8 h
		LTL-C		
5	75	38	1.6 min	0.1 min
6	75	38	1.6 h	15.0 min

[a] The $t_{1/2}$ values include the delay time for initiation of precipitation.

Table IV: Electron Microscopic Patterns of the Precipitates of LTL-A in Mixed-Oligosaccharide Precipitation Systems

mixture of	concn of LTL-A (μM)	oligosacch total concn (μM)	molar ratio of oligosacch	incubation time (h)	pattern[a]
5 and **6**	130	70	50:50	24	c
	130	70	50:50	0.5	none
	130	135	50:50	24	c
	100	60	70:30	24	c
	100	60	80:20	24	b
	100	60	90:10	24	b
5 and **7**	130	60	50:50	96	b
	130	130	50:50	96	b
	130	130	50:50	0.5	b
	130	210	50:50	96	b
	140	120	20:80	96	b
	130	110	10:90	96	b (diffused)
	100	90	5:95	96	d

[a] The letters b, c, and d represent patterns b, c, and d in Figure 5, respectively.

reported previously (Bhattacharyya et al., 1989b) and is included here for comparison. A comparison between parts a and b of Figure 5 shows that the patterns obtained by two different electron microscopic methods are similar; both consist of bands of "zipperlike" filamenteous structures. In addition, the pattern disappears in the presence of fucose, but nonbinding sugars, such as mannose and galactose, have no effect. Furthermore, neither LTL-A nor oligosaccharide **5** alone show any pattern under identical conditions. The results show that the patterns are intrinsic and not artifacts of sample processing. Parts c and d of Figure 5 show the results of electron microscopy of the negatively stained precipitates of LTL-A in the presence of oligosaccharides **6** and **7**, respectively. As with **5**, the patterns disappear on addition of fucose to the precipitates. Mannose and galactose have no effect.

In the above studies, precipitates were formed by using equivalent concentrations (in terms of binding sites) of LTL-A and the oligosaccharides. When precipitates from different data points across the precipitation profile (Figure 2) were examined, similar images were obtained.

While the precipitates of LTL-A with the oligosaccharides showed unique long-range ordered lattices, the precipitates of LTL-C with **5**–**7** did not demonstrate any pattern, either by negative-stain or freeze-fracture methods.

Electron Microscopy of Precipitates of LTL-A with Binary Oligosaccharide Mixtures. Table IV summarizes the results of electron microscopy of the negatively stained precipitates of LTL-A in the presence of binary mixtures of oligosaccharides **5** and **6** and **5** and **7**. Binary mixtures of **5** and **6** show the pattern characteristic of either one or the other oligosaccharide, depending on the ratios of their concentrations and time of incubation. The pattern of the precipitates remained the same over a range of different total oligosaccharide to protein concentrations (Table IV). The time of incubation

Table V: Electron Microscopic Patterns of the Precipitates of Isolectin Mixtures in the Presence of Oligosaccharide **5**

mixture	total protein concn (μM)	molar ratio of isolectins	oligosacch **5** concn (μM)	pattern[a,b]
LTL-A and LTL-B	140	50:50	70	b
LTL-A and LTL-C	130	50:50	70	none
	130	70:30	70	none
	130	80:20	70	b
LTL	110		55	none

[a] The incubation time is 20–24 h in all cases. [b] The letter b represents the pattern in Figure 5b.

of the protein with an equimolar mixture of **5** and **6** affected the observed pattern of the precipitates. After 30 min, the precipitates of LTL-A and an equimolar mixture of **5** and **6** showed no pattern. However, after 24 h the pattern for **6** was observed in the mixture (similar to that in Figure 5c). The latter result was also found with a 70:30 molar ratio of the two oligosaccharides. However, an 80:20 mixture of oligosaccharides **5** and **6** produces a zipperlike pattern characteristic of **5**.

The precipitates of LTL-A with an equimolar mixture of oligosaccharides **5** and **7** show a pattern characteristic of **5** after both short and long incubation times. The observed pattern did not change when using different total concentrations of the protein and oligosaccharides (Table IV). The pattern for **5** was also seen with the precipitates obtained from molar ratios of **5** and **7** as high as 10:90 in the presence of LTL-A; however, the tertiary organization is much less well-defined. With a 5:95 mixture of the oligosaccharides, the zipperlike pattern of **5** is absent, and the pattern is like that of **7** (Table IV).

Electron Microscopy of the Precipitates of Mixed LTL Isolectins with Oligosaccharide **5**. Table V summarizes the EM results of the precipitates of mixtures of the isolectins in the presence of oligosaccharide **5**. The results show that the pattern of LTL-A and **5** remains unaffected in the presence of an equimolar amount of LTL-B. However, an equimolar mixture of LTL-A and LTL-C fails to show any pattern, as does a 70:30 mixture of the two isolectins. An 80:20 ratio of LTL-A and LTL-C, on the other hand, shows the pattern of LTL-A with **5**.

The native mixture, which is composed of approximately 50%, 35%, and 15% of LTL-A, LTL-B, and LTL-C, respectively [cf. Goldstein and Poretz (1986)], does not show a pattern with oligosaccharide **5**, which is consistent with the lack of an observed pattern with 50:50 and 70:30 mixtures of LTL-A and LTL-C.

Discussion

Binding Specificities of LTL-A, LTL-B, and LTL-C with Fucosyl Oligosaccharides. Previous studies have reported that native mixtures of *Lotus tetragonolobus* isolectins bind primarily to α-fucose residues in oligosaccharides [cf. Goldstein and Poretz (1986)]. Differences in binding affinities of the three isolectins toward fucose and 2′-*O*-α-fucosyllactose were reported (Pereira & Kabat, 1974). The results of the present study, however, show that all three isolectins have essentially the same affinities for oligosaccharides **1**–**6** as for fucose. Interestingly, the presence of two α-fucose residues in oligosaccharides **4**–**6** does not lead to enhanced binding relative to fucose. This result contrasts with the observation that an increased number of binding residues in certain asparagine-linked oligosaccharides leads to enhanced binding to Con A (So & Goldstein, 1968; Bhattacharyya et al., 1987b; Bhat-

tacharyya & Brewer, 1989) and certain galactose-specific lectins (Bhattacharyya et al., 1988a). The fact that LTL-A shows weaker affinities for oligosaccharides **7** and **8** suggests that the isolectin is sensitive to the structure of the core region of the carbohydrates.

Although the presence of galactose residues in oligosaccharides **2** and **6** has little effect on the affinity of the LTL isolectins for the carbohydrates, the presence of the fucose residues has a significant effect on binding of the oligosaccharides to certain galactose-specific lectins. The defuco analogues of oligosaccharides **2** and **6** bind to many galactose-specific lectins including EIL and RCA-I (Bhattacharyya et al., 1988a). However, the presence of $\alpha(1-3)$ fucose in **2** and **6** completely abolishes binding to these lectins. This suggests a possible regulatory role for the fucose in these oligosaccharides in terms of modulating the binding activities of galactose-specific lectins, as well as establishing binding to fucose-specific lectins. This may be particularly important in view of altered expression of the Le^x antigenic determinant, Galβ(1–4)[Fucα(1–3)]GlcNAc, during a variety of biological processes (Sharon, 1983; Muramatsu, 1982; Glabe et al., 1982; Hakomori & Kannagi, 1983; Hakomori, 1984) including many pathological conditions (Santer et al., 1983; Hakomori, 1984; Kannagi et al., 1986; Itzkowitz et al., 1986). Interestingly, fucose linked $\alpha(1-2)$ to galactose does not interfere with the binding of EIL to oligosaccharide **3**, although it prevents binding of RCA-I because a free C-2 hydroxyl group is required for the binding of the protein to galactose (Bhattacharyya & Brewer, 1988).

Precipitation Activities of LTL-A and LTL-C with Fucosyl Oligosaccharides. Oligosaccharides **5**–**7** also precipitate with LTL-A and LTL-C. Their precipitation profiles (Figures 2 and 3) are similar to those observed for Con A and certain galactose-specific lectins with multiantennary oligosaccharides and glycopeptides (Bhattacharyya et al., 1987a,b, 1988a, 1989; Bhattacharyya & Brewer, 1989) and indicate similar multivalent interactions in the complexes.

The ratio of concentrations of oligosaccharide at the equivalence point to the concentration of protein monomer is approximately 1:2 for oligosaccharides **5** and **6** with both LTL-A and LTL-C (Table II). Since both isolectins are tetramers [cf. Goldstein and Poretz (1986)], the results give a stoichiometry of 2:4 for the precipitation reactions. Both isolectins possess one binding site per monomer (Kalb, 1968), and thus oligosaccharides **5** and **6** are each divalent and can bind and cross-link two separate protein molecules via their two terminal fucose residues.

Oligosaccharide **7**, which has approximately 5-fold weaker affinity than oligosaccharides **5** and **6** (Table I), has an oligosaccharide to LTL-A monomer stoichiometry of 1:1.3. The fact that **7** can cross-link and precipitate with LTL-A indicates that the oligosaccharide must be divalent (Kabat, 1976). However, due to the weak affinity of the carbohydrate the equivalence point is shifted toward the carbohydrate excess region to give an apparent stoichiometry of less than 1:2. Similar results have been found in the precipitation profiles of Con A with certain oligomannose-type glycopeptides (Bhattacharyya & Brewer, 1989) and the soybean lectin with complex-type oligosaccharides (Bhattacharyya et al., 1988a).

Oligosaccharides **1**–**3** form only soluble complexes with LTL-A and LTL-C, as expected. Oligosaccharides **4** and **8**, which possesses two fucose residues, also do not precipitate with the isolectins. Thus, although **4** and **8** each possess two fucose residues, they are unable to cross-link the isolectins. Furthermore, even though oligosaccharides **7** and **8** are isomeric and bind to LTL-A with essentially the same affinities, only **7** precipitates with the protein. The reason for this difference in precipitating activities between **7** and **8** is apparent from an inspection of Corey–Pauling–Koltun (CPK) models of the two oligosaccharides (Figure 6). Both molecules are shown with their $\beta(1-6)$ arms in the $\omega = -60°$ rotamer conformation, which is one of the two allowed conformations for this arm in solution [cf. Brisson and Carver (1983)]. This is also the bound conformation of **7** in its cross-linked complex with LTL-A as determined from electron microscopy and X-ray diffraction data (Brewer and Makowski, unpublished results). Both fucose residues in **7** (Figure 6a) are widely spaced from each other and capable of binding two separate lectin molecules. However, the CPK model of **8** (Figure 6b) in either allowed conformation of the $\beta(1-6)$ arm shows that the two fucose residues are at nearly right angles to each other and therefore unable to accommodate two lectin molecules for cross-linking. These results indicate that the position and linkage of fucose residues in the oligosaccharides are critical for cross-linking with the proteins.

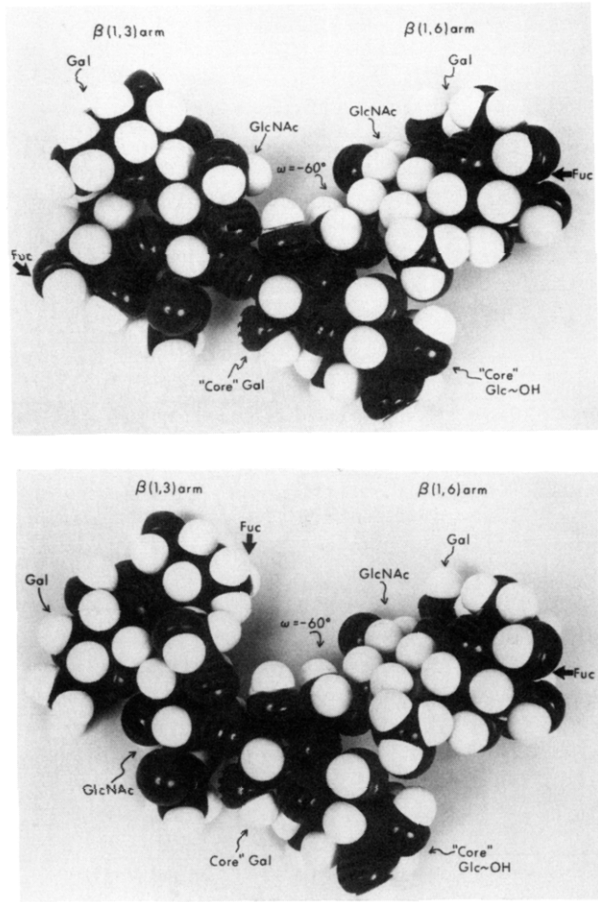

FIGURE 6: Corey–Pauling–Koltun space-filling models of oligosaccharides **7** (top) and **8** (bottom) with the rotation angle of the $\beta(1-6)$ arm set to $\omega = -60°$. The angle, ω, is the dihedral angle formed by the H-5, C-5, C-6, and O-6 atoms of the core β-galactose residue. Fuc, Gal, GlcNAc, and GlcOH represent fucose, galactose, *N*-acetylglucosamine, and glucose residues, respectively, in the oligosaccharides.

The oligomeric structures of the isolectins are also an important determinant in the precipitation reactions. Only LTL-A and LTL-C, which are tetramers, undergo precipitation with oligosaccharides **5**–**7**, while LTL-B, which is a dimer, does not precipitate even though it possesses essentially the same affinity for the oligosaccharides. These findings are consistent with previous observations that certain biantennary complex-type oligosaccharides containing galactose residues

at both nonreducing termini precipitate with tetrameric but not dimeric galactose-specific lectins (Bhattacharyya et al., 1988a). Similar results have been reported for antigen–antibody precipitation reactions [cf. Kabat (1976)].

The present findings also demonstrate that the absence or presence of galactose residue(s) in oligosaccharides **5** and **6**, respectively, has dramatic effects on the temperature dependence and kinetics of the precipitation reactions of LTL-A, even though the isolectin possesses essentially the same binding affinity for both oligosaccharides.

Electron Microscopic Studies of the Precipitates of LTL-A and LTL-C with Oligosaccharides **5**–**7**. The negative-stain and freeze-fracture electron micrographs show that the precipitates of LTL-A with oligosaccharides **5**–**7** each possess unique lattice patterns that are characteristic of crystalline solids. The fact that a single unique lattice geometry is observed for each cross-linked complex indicates that bound oligosaccharide must be rigidly held in each lattice. Thus, even though oligosaccharides **5** and **6** possess essentially the same affinity (Table I) and very similar precipitation profiles (Figure 2) to LTL-A, each forms a different cross-linked lattice with the protein, which indicates different bound conformations for the oligosaccharides. Furthermore, although **6** and **7** possess the same binding moiety, the Le^x blood group antigenic determinant, the presence of different core structures in the oligosaccharides results in different lattice patterns with LTL-A.

Electron micrographs of negatively stained and freeze-fracture preparations of the precipitates of LTL-C with oligosaccharides **5**–**7**, on the other hand, did not show any pattern. Since the precipitates of LTL-C were formed by specific binding and cross-linking interactions with the oligosaccharides, as with LTL-A, they must form specific lattices. However, it appears that the lattices formed with LTL-C may not be stable enough to be visualized by electron microscopy. This is consistent with weaker precipitation of LTL-C in the presence of oligosaccharides **5** and **6** compared to LTL-A (Figures 2 and 3). These results show that although the two isolectins possess similar affinities for **5**–**7**, they possess different precipitating activities.

Electron Microscopic Studies of Binary Mixtures of the Oligosaccharides in the Presence of LTL-A. The time-dependent formation of the pattern of the precipitates in an equimolar mixture of **5** and **6** with LTL-A (Table IV) appears to be due to a combination of the kinetic and thermodynamic properties of binding and precipitation of the oligosaccharides with the protein. Precipitation of **5** with LTL-A is essentially complete in less than 10 min whereas oligosaccharide **6** requires approximately 4 h for the onset of precipitation (Table III). Thus, precipitation of **5** is nearly complete before precipitation of **6** begins. It appears, therefore, that the existance of a relatively high concentration of soluble complexes of **6** with LTL-A interferes with the long-range growth of the lattice of **5** with the protein. However, with most of **5** precipitated within 4 h, the growth of the lattice of **6** is unimpeded and is observed after 24 h. This also explains the results obtained with a 70:30 mixture of **5** and **6**, which also shows only the pattern for **6**. However, with an 80:20 ratio of **5** and **6**, the concentration of LTL-A complexes with **6** is apparently too low to inhibit the long-range growth of the lattice of **5**, and thus the pattern of **5** is observed while that for **6** is not.

These results are different from that observed for binary mixtures of oligosaccharides **5** and **7** with LTL-A. The precipitates of the protein with an equimolar mixture of **5** and **7** show the pattern characteristic of **5** after both 30 min and 96 h. While the affinity of LTL-A for **5** is 5-fold higher than that for **7** in soluble complexes (Table I), the ratio of affinities of the two oligosaccharides in their respective cross-linked states with LTL-A is not known. However, since cross-linking involves multiple interactions between lectin and oligosaccharide molecules, the ratio of affinities of **5**–**7** must be greater than 5:1 in their respective cross-linked complexes. Therefore, the proportion of cross-linked complexes of LTL-A with **5** in the mixture is much higher than that with **7**, which explains why the pattern of **5** is observed. However, at a 5:95 ratio of the two oligosaccharides, only the pattern for **7** is observed due to the relatively high concentration of its complexes with LTL-A.

These results demonstrate that only the cross-linked lattice patterns for individual oligosaccharides with LTL-A can be observed in electron micrographs in the presence of mixtures of the carbohydrates, but not hybrid patterns. This provides additional support for the conclusion that each oligosaccharide forms a homogeneous cross-linked lattice with the protein that excludes the other oligosaccharides.

Electron Microscropic Studies of Binary Mixtures of the LTL Isolectins with the Oligosaccharides. An equimolar mixture of LTL-A and LTL-B in the presence of oligosaccharide **5** shows that the latter isolectin, which cannot cross-link with the oligosaccharide, does not interfere with the formation of the lattice pattern of LTL-A and **5**. However, LTL-C, which can cross-link with **5**, does interfere with the long-range growth of the lattice of LTL-A and **5**. These results demonstrate that inhibition of the long-range growth of an LTL-A cross-linked lattice with an oligosaccharide requires competitive binding at the cross-linking level by another isolectin (LTL-C). This suggests that nucleation of an oligosaccharide–lectin complex can inhibit the long-range lattice growth of another competitive oligosaccharide–lectin cross-linked complex with the same lectin. These findings are consistent with the relative inhibition of lattice growth observed above for binary mixtures of the oligosaccharides and LTL-A.

Conclusions. Using quantitative precipitation analysis, we have recently provided evidence that ConA forms unique homogeneous cross-linked lattices with a variety of bivalent oligomannose and bisected hybrid type glycopeptides (Bhattacharyya et al., 1988b). This suggested the presence of long-range order in the precipitates formed between such multivalent carbohydrates and lectins. In a preliminary electron microscopic study, we have shown that the precipitates formed between the *N*-acetylgalactosamine/galactose-specific lectin from soybean (*Glycine max*) and a biantennary complex type oligosaccharide containing terminal galactose and the precipitates formed between LTL-A and oligosaccharide **5** each possess highly organized and unique lattice structures (Bhattacharyya et al., 1989b).

In the present study, we show that a single lectin (LTL-A) forms unique homogeneous cross-linked complexes with three structurally related bivalent oligosaccharides. The present findings also provide insight into the molecular interactions responsible for the formation of these cross-linked complexes in that each complex resembles a different crystalline array. Thus, the unique molecular packing interactions that stabilize each lattice appear to be the basis for the specificity of formation of homogeneous cross-linked lectin–carbohydrate complexes. Heterogeneous complexes involving two different oligosaccharides bound to a lectin do not precipitate presumably due to the thermodynamic instability of such lattices.

The present findings provide support for our recent observations of a new source of specificity in the interactions of

multivalent oligosaccharides and lectins, namely, the formation of homogeneous cross-linked complexes. These findings may relate to the functions of the oligosaccharides as putative receptors on cell surfaces, since it is known that cross-linking of cell-surface carbohydrate receptors is a key step in many biological events (Nicolson, 1974; Brown & Hunt, 1978; DeLisi & Blumenthal, 1979; Lennarz, 1980; Monsigny, 1984; Fenderson et al., 1984; Brandley & Schnaar, 1986). Thus, these studies provide models of the spontaneous formation of homogeneous aggregates between glycoconjugates and lectins. Furthermore, our results suggest the possibility of distinct functional roles for certain isolectins. Lastly, the presence of highly organized well-defined lattices for oligosaccharide–lectin complexes will allow an analysis of the lattice geometries of individual complexes by a combination of electron microscopic and X-ray diffraction studies and thereby provide structural information of the molecules in their bound states.

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