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Binding of Multivalent Carbohydrates to Concanavalin A and *Dioclea grandiflora* Lectin

THERMODYNAMIC ANALYSIS OF THE "MULTIVALENCY EFFECT"*

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Binding of a series of synthetic multivalent carbohydrate analogs to the Man/Glc-specific lectins concanavalin A and *Dioclea grandiflora* lectin was investigated by isothermal titration microcalorimetry. Dimeric analogs possessing terminal α-*D*-mannopyranoside residues, and di-, tri-, and tetrameric analogs possessing terminal 3,6-di-*O*-(α-D-mannopyranosyl)-α-D-mannopyranoside residues, which is the core trimannoside of asparagine-linked carbohydrates, were selected in order to compare the effects of low and high affinity analogs, respectively. Experimental conditions were found that prevented precipitation of the carbohydrate-lectin cross-linked complexes during the isothermal titration microcalorimetry experiments. The results show that the value of *n*, the number of binding sites on each monomer of the lectins, is inversely proportional to the number of binding epitopes (valency) of each carbohydrate. Hence, *n* values close to 1.0, 0.50, and 0.25 were observed for the binding of mono-, di-, and tetravalent sugars, respectively, to the two lectins. Importantly, differences in the functional valency of a triantennary analog for concanavalin A and *D. grandiflora* lectin are observed. The enthalpy of binding, Δ*H*, is observed to be directly proportional to the number of binding epitopes in the higher affinity analogs. For example, Δ*H* of a tetravalent trimannoside analog is nearly four times greater than that of the corresponding monovalent analog. Increases in K_a values of the multivalent carbohydrates relative to monovalent analogs, known as the "multivalency effect," are shown to be due to more positive entropy ($T\Delta S$) contributions to binding of the former sugars. A general thermodynamic model for distinguishing binding of multivalent ligands to a single receptor with multiple, equal subsites *versus* binding to separate receptor molecules is given.

Carbohydrate-protein interactions are involved in a wide variety of biological functions including cellular growth, recognition, adhesion, cancer metastasis, bacterial and viral infections, and inflammation (1, 2). The specificity of these interactions has been an active area of research due, in part, to efforts at designing therapeutic analogs of carbohydrates (3, 4). However, attempts to design high affinity analogs for specific carbohydrate-binding proteins (lectins) have been difficult due to the intrinsic low affinity of carbohydrates in many cases (5, 6). For example, the affinity constants (K_a) for the binding of simple mono- and oligosaccharides to most lectins are between 10^3 and 10^6 M^{-1} (7, 8). This range of K_a values is too low for effective drug design. However, many naturally occurring carbohydrates and glycoconjugates including glycoproteins and glycolipids are multivalent (2) which results in their increased avidity for lectins (9). As a consequence, there has been considerable interest in designing multivalent or "clustered" carbohydrate analogs for high affinity binding to target lectin receptors (10, 11). Thus, it is important to understand the thermodynamic basis of the enhanced binding of multivalent glycoconjugates to lectins.

Binding of multivalent carbohydrates (and glycoconjugates) to lectins, which themselves are generally multivalent (2), may or may not lead to the formation of lectin-carbohydrate cross-linked complexes (12). This depends on the valencies of the two molecules, and the structural arrangement of the carbohydrate epitopes and binding sites (subunits) of the lectin. In cases where cross-linking occurs, several different types of complexes can result. When both the carbohydrate and lectin are divalent, one-dimensional soluble cross-linked complexes may form. However, when the valencies of the carbohydrate and lectin are greater than two for either molecule, two- and three-dimensional cross-linking can occur resulting in highly ordered complexes. In the latter case, precipitation of the ordered cross-linked complex generally occurs, and occasionally crystals of the complex may result. This type of cross-linked complex has been extensively described by Brewer and co-workers (12, 13). Importantly, high affinity binding occurs in this type of cross-linked complex since both the carbohydrate and lectin simultaneously bind to neighboring molecules in the lattice.

Multivalent binding between carbohydrates and lectins can also occur without cross-linking interactions, as in the case where the binding sites of a lectin are clustered together or face the same direction (14). An example of clustered binding sites is the hepatic asialoglycoprotein receptor (9). Binding of a monoantennary complex oligosaccharide gives an inhibition constant, $(I)_{50}$, of $\sim 10^3$ M^{-1}, while a triantennary complex oligosaccharide possesses an $(I)_{50}$ of $\sim 10^9$ M^{-1} (15). The presence of subsites for each arm of the trivalent oligosaccharide on three clustered subunits of the receptor is known to be responsible for the large increase in the affinity of the triantennary carbohydrate (9). The $\sim 10^6$ increase in affinity of the trivalent carbohydrate relative to the monovalent sugar is essentially the theoretical limit of the sum of the exponentials of the

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binding constants of the individual subsites (*i.e.* $10^3 \times 10^3 \times 10^3 = 10^9$).

More recently, clustered or "dendritic" multivalent carbohydrate analogs have been synthesized in order to obtain enhanced affinity for lectins with binding sites well separated on adjacent monomers of the protein. For example, clustered or dendritic GlcNAc-based synthetic analogs with valencies of two, four, and eight were prepared on a scaffolding of L-lysine (16). The binding potencies of the dendrimers derived from IC_{50} mM values for inhibiting wheat germ agglutinin binding to porcine stomach mucin relative to a monomeric analog were 5-fold for the divalent derivative, 25-fold for the tetrameric derivative, and 170-fold for the octameric derivative. Thus, significant enhancements in the affinities of multivalent carbohydrates for multivalent lectins have been observed. However, the thermodynamic basis for these increased affinities are not understood.

Isothermal titration microcalorimetry (ITC)[1] has recently been used to study the binding interactions between carbohydrates and lectins. ITC measurements provide direct determinations of the value of n, the number of binding sites of the protein, ΔH, the enthalpy of binding, and K_a, the association constant. From measurements of K_a, the free energy of binding, ΔG, can be calculated, and hence the entropy of binding, ΔS, determined. Thus, ITC measurements can determine the complete thermodynamics of binding of a carbohydrate to a lectin. As examples, the binding of mono- and oligosaccharides to the Man/Glc-pecific lectin concanavalin A (ConA) and the seed lectin from *Dioclea grandiflora* (DGL) have been investigated using ITC measurements (8, 17–19). A large increase in the -ΔH value of 3,6-di-*O*-(α-D-mannopyranosyl)-α-D-mannopyranose, which is present in the "core" region of all asparagine-linked carbohydrates, relative to that of methyl-α-D-mannopyranoside (Me-αMan), for both lectins suggested extended binding sites in both proteins for the trimannoside (19). Importantly, these results were confirmed by x-ray crystallographic studies of ConA (20) and DGL (21). Thus, ITC measurements can provide thermodynamic as well as structural information on carbohydrate-lectin interactions. However, to date ITC measurements have not extensively been applied to the study of multivalent carbohydrate-lectin interactions.

In the present study, we report ITC measurements of the binding of synthetic multivalent carbohydrates to ConA and DGL. Synthetic dimeric analogs of α-D-mannopyranoside (Figs. 1 and 2) and di-, tri-, and tetrameric analogs of 3,6-di-*O*-(α-D-mannopyranosyl)-α-D-mannopyranoside (Figs. 3 and 4) were used in the study. The results show that ITC can be used to determine the functional valence of multivalent carbohydrates for ConA and DGL, and the thermodynamic basis for the enhanced affinities of the multivalent sugars.

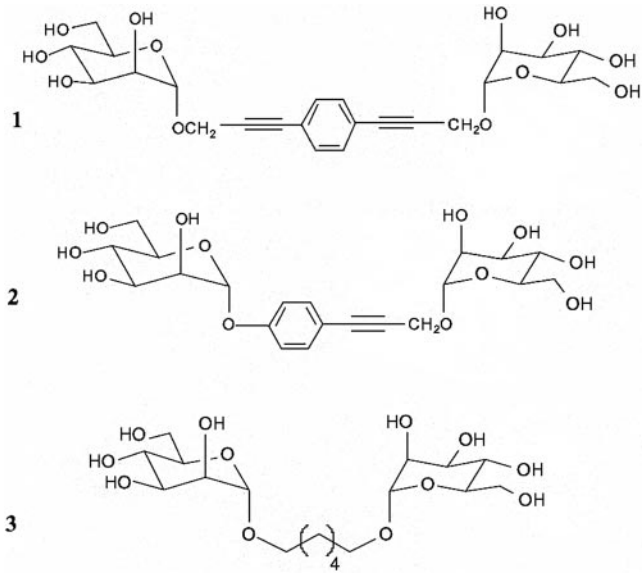


FIG. 1. **Structures of multivalent analogs 1–3**.

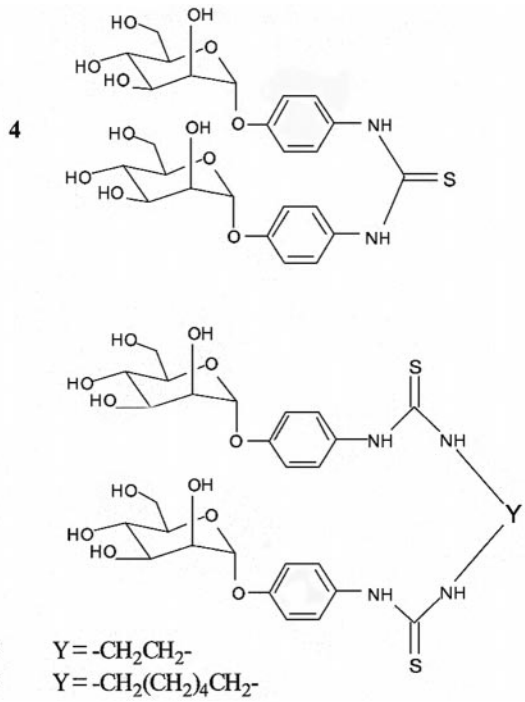


FIG. 2. **Structures of multivalent analogs 4–6**.

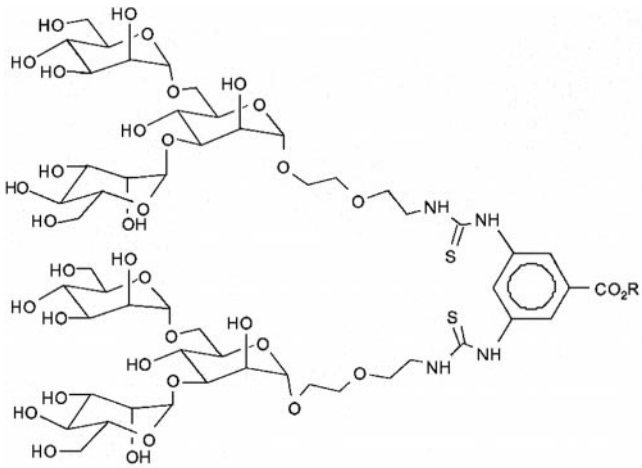
MATERIALS AND METHODS

DGL was isolated from *D. grandiflora* seeds obtained from North Eastern Brazil (Albano Ferreira Martin Ltd., Rua Teodoreto Souto, 718 (cambuci) 01634-000 Sao Paulo, Brazil) as described previously (22). The concentration of DGL was determined spectrophotometrically at 280 nm using $A^{1\%}_{1\ cm} = 12.0$ at pH 5.2 and expressed in terms of monomer (M_r = 25,000) (22). ConA was prepared from Jack bean (*Canavalia ensiformis*) seeds (Sigma) according to the method of Agrawal and Goldstein (23). The concentration of ConA was determined spectrophotometrically at 280 nm using $A^{1\%}_{1\ cm} = 12.4$ at pH 5.2 (24) and expressed in terms of monomer (M_r = 25, 600).

Me-αMan, *p*-aminophenyl-α-D-mannopyranoside, *p*-nitrophenyl-α-D-mannopyranoside and methyl-3,6-di-*O*-(α-D-mannopyranosyl)-α-D-mannopyranoside (Tri-Man), were purchased from Sigma. The synthesis of carbohydrate analogs **1** and **2** has previously been reported (25), as have analogs **4, 5,** and **6** (26). The synthesis of analogs **7, 8, 9,** and **10** has also been previously described (27). Synthesis of **3** will be reported elsewhere. The concentrations of the carbohydrates were determined by modification of the Dubois phenol/sulfuric acid method using appropriate monosaccharides as standards (28).

Isothermal Titration Microcalorimetry—ITC experiments were performed using a model MCS instrument from Microcal, Inc. (Northampton, MA). Injections of 4 μl of carbohydrate solution were added from a computer controlled 250- or 100-μl microsyringe at an interval of 4 min into the sample solution of lectin (cell volume = 1.358 ml) with stirring at 350 rpm. An example of an ITC experiment is shown in Fig. 5 for tetravalent trimannoside **10** with ConA at 27 °C. Control experiments performed by making identical injections of saccharide into a cell containing buffer without protein showed insignificant heats of dilution. The concentration of lectins were 0.015–0.14 mM and the sugars were 0.16–4.0 mM, respectively. Titrations were done at pH 5.0–5.2 and at NaCl concentrations from 0 to 0.15 M. The experimental data were

[1] The abbreviations used are: ITC, isothermal titration microcalorimetry; ConA, lectin from Jack bean (*Canavalia ensiformis*); DGL, seed lectin from *D. grandiflora*; Me-αMan, methyl-α-D-mannopyranoside, Tri-Man,methyl-3,6-di-*O*-(α-D-mannopyranosyl)-α-D-mannopyranoside; all sugars are in the D-configuration.



7 R = H
8 R = Me

FIG. 3. **Structures of multivalent analogs 7 and 8**.

fitted to a theoretical titration curve using software supplied by Microcal, with ΔH (enthalpy change in kcal mol^{-1}), K_a (association constant in M^{-1}) and n (number of binding sites per monomer), as adjustable parameters. The quantity $c = K_a M_t(0)$, where $M_t(0)$ is the initial macromolecule concentration, is of importance in titration microcalorimetry (29). All experiments were performed with c values $1 < c < 200$. The instrument was calibrated using the calibration kit containing ribonuclease A (RNase A) and cytidine 2′-monophosphate supplied by the manufacturer. Thermodynamic parameters were calculated from the equation,

$$\Delta G = \Delta H - T\Delta S = -RT \ln K_a \qquad \text{(Eq. 1)}$$

where ΔG, ΔH, and ΔS are the changes in free energy, enthalpy, and entropy of binding. T is the absolute temperature and R = 1.98 cal mol^{-1} K^{-1}.

RESULTS AND DISCUSSION

Previous ITC studies have shown that values of n, the number of binding sites per monomer of protein, for simple mono- and oligosaccharides binding to ConA (8) and DGL (19) are close to 1.0 for both lectins. For example, the values of n are 1.0 for the binding of both Me-αMan and Tri-Man to ConA (Table I) and DGL (Table II). These values agree with the x-ray crystal data for the number of binding sites on each monomer of ConA for Me-αMan (30) and 3,6-di-*O*-(α-D-mannopyranosyl)-α-D-mannopyranose (20), and of DGL for Tri-Man (21).

However, n values less than 1.0 have been reported for the binding of multivalent carbohydrates to ConA. An n value of 0.59 was reported by Brewer and co-workers (8) for the binding of a Man9 oligomannose glycopeptide to acetyl-ConA, which is a dimer at pH 7.0 (31). An n value of 0.64 was also reported for the binding of a Man5 oligomannose to acetyl-ConA (8). Both of these carbohydrates were previously shown to be divalent for ConA (32), with one binding site on the α(1–3) arm of the core Man residue, and the second site on the α(1–6) arm of the core Man residue. Both carbohydrates are also known to bind and precipitate tetrameric ConA at pH 7.2 (32). In addition, the Man5 oligosaccharide has also been shown to bind and precipitate with tetrameric DGL at pH 7.2 (33). Brewer and co-workers (8) concluded that the relatively low n values associated with binding of the Man5 and Man9 carbohydrates to acetyl-ConA were due to the formation of soluble one-dimensional cross-linked complexes between the divalent lectin and the divalent carbohydrate(s).

ITC derived values of n less than 1.0 have also been reported for the binding of synthetic divalent analogs to ConA. The n values for binding of two divalent C-glycoside analogs possessing terminal Glc or Man residues to tetrameric ConA and succinyl-ConA, which is also reported to be a dimer (31), were reported to be close to 0.50 for both forms of the lectin (34). The results in the present study suggest that divalent cross-linking by the sugars is responsible for their low n values.

Structures of the Carbohydrate Analogs—Synthetic analogs **1–6** in Figs. 1 and 2 possess terminal α-D-mannopyranoside residues in each molecule. The structures of dimeric analogs **1**-**3** differ in terms of the flexibility and length of the spacer groups in each molecule. Analog **1** has a relatively rigid, linear spacer group, a phenyl ring with *para*-substituted acetylenic groups, between the two terminal Man residues. Analog **2** possesses a shorter, rigid, linear spacer group, with the absence of one of the acetylene groups found in **1**. Analog **3** possesses a hexyl chain as a spacer group, and hence is close to the length of **2** but more flexible than either **1** or **2**.

The structures of analogs **4**-**6** differ from those of **1**-**3** in having even longer spacer groups. These groups are derived from a *para*-substituted phenylthiourea group that is connected to ethyl and hexyl chains in **5** and **6,** respectively. Thus, the distance separating the terminal Man residues in **4**-**6** is progressively increased.

Synthetic analogs **7–10** in Figs. 3 and 4 possess terminal 3,6-di-*O*-(α-D-mannopyranosyl)-α-D-mannopyranoside residues. Previous ITC studies have shown that the individual trimannoside epitope (Tri-Man) binds with much higher affinity than Me-αMan to ConA (8) and DGL (19), as shown in Tables I and II, respectively. The spacer groups in analogs **7** and **8** are based on a 3,5-disubstituted benzoic acid ring in which the two thiourea moieties on the phenyl ring are coupled to glycosidically linked alkyl ether chain. Thus, **7** and **8** are biantennary analogs of the trimannoside moiety, differing only in a methyl group on the benzoic acid moiety (ester) of **8**. Analog **9** possesses three thiourea-substituted chains terminating in the trimannoside moiety. Analog **10** possesses two trisubstituted phenyl rings with each ring possessing two thiourea-linked alkyl either chains coupled to a terminal trimannoside moiety. Hence, **10** possesses four terminal trimannoside groups.

Experimental Conditions for ITC Measurements—At pH 7.2 and NaCl concentrations greater than 0.15 M where ConA and DGL are tetramers, analogs **1**-**10** are observed to bind and precipitate with both proteins at lectin concentrations between 25 and 60 μM and at nearly stoichiometric ratios of the sugars. Quantitative precipitation profiles of the lectins with the sugars confirmed their ability to precipitate the proteins (data not shown). The precipitation reactions were inhibited by the presence of Me-αMan or dissolved upon addition of the monosaccharide. Thus, the multivalent binding activities of the sugars were confirmed, since tetrameric ConA (12) and tetrameric DGL (33) are known to precipitate with multivalent carbohydrates and glycoproteins. ITC measurements, however, require the presence of soluble complexes during the titration experiment (~1–2 h), and thus conditions in which the sugar complexes with ConA and DGL remained soluble were investigated.

The dimer-tetramer equilibrium of ConA has been reported to be sensitive to pH, so that the tetramer exist at pH 7 while the dimer is reported to predominate at pH 5.0 (35, 36). Acetylation or succinylation of the amino groups of ConA is reported to produce dimeric derivatives of ConA (31). We have observed that at concentrations between 15 and 140 μM ConA and DGL, both lectins at room temperature are dimers at pH 5.0 and low salt concentrations (less than 0.15 M NaCl).[2] Under these con-

[2] T. K. Dam, R. Roy, S. K. Das, S. Oscarson, and C. F. Brewer, unpublished results.

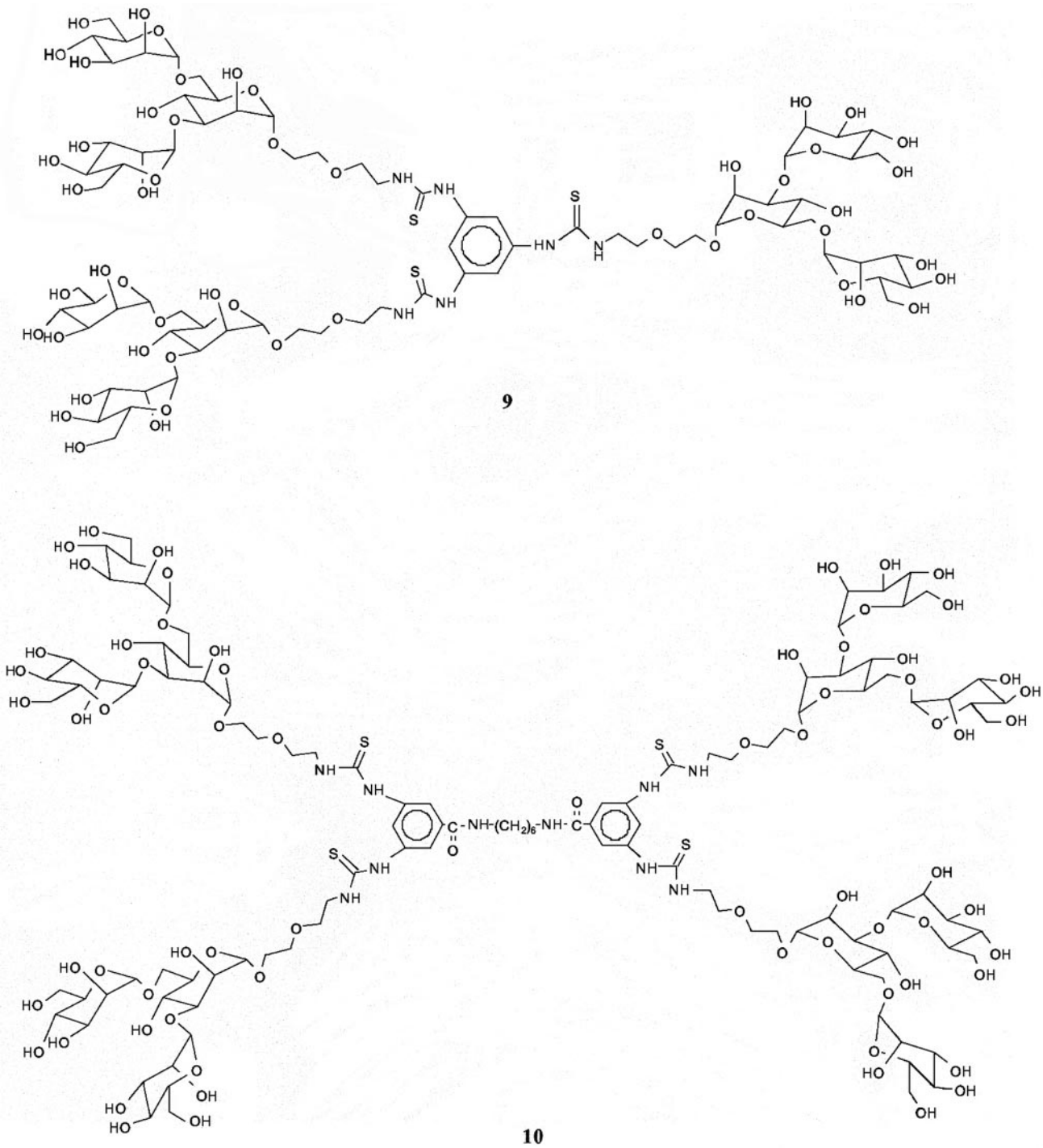


FIG. 4. **Structures of multivalent analogs 9 and 10**.

ditions, precipitation of both lectins by analogs **1**-**10** either is not observed or considerably slowed. Dimeric ConA and DGL do not precipitate with divalent sugars **1**-**7,** since they apparently form linear cross-linked complexes with the sugars that are soluble. On the other hand, analogs **9** and **10** are potentially tri- and tetravalent, respectively, and capable of forming two- and three-dimensional cross-linked complexes even with dimeric ConA and DGL. However, using concentrations of ~15 μM ConA and DGL at pH 5.0 and low salt concentration, complexes of **9** and **10** with both lectins are observed to be stable during the ~1 h required for the ITC experiments. Time-dependent precipitation of the complex occurred after the run. Thus, the ITC experiments reported in the present study were performed under conditions in which cross-linked complexes between the sugars and lectins were soluble.

ITC Measurements of K_a and n Values of the Multivalent Carbohydrates—Tables I and II show data for the binding of **1–10** to ConA and DGL, respectively. For comparison, data for the binding of Me-αMan, *p*-aminophenyl-α-D-mannopyranoside, and Tri-Man, which represent the monovalent binding epitopes of the respective analogs, are also shown. As can be seen, dimeric analogs **1**-**6** with terminal α-D-mannopyranoside residues possess much lower affinities for the two lectins than Tri-Man and dimeric analogs **7** and **8** which possess two trimannoside moieties. Tri- and tetraantennary analogs **9** and **10** have even higher affinities for both lectins. These results are

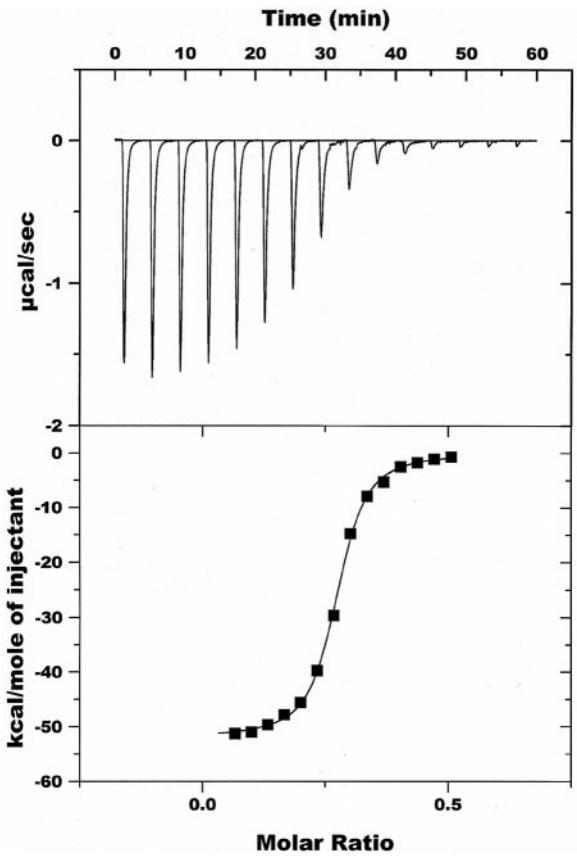


FIG. 5. **ITC profile of ConA (0.020 mM) with analog 10 (0.24 mM) at 27 °C.** *Top,* data obtained for 30 automatic injections, 4 μl each, of **10**; *bottom,* the integrated curve showing experimental points (■) and the best fit (—). The buffer was 0.1 M sodium acetate at pH 5.2 with 0.1 M NaCl, 5 mM each of $CaCl_2$ and $MnCl_2$.

TABLE I
Thermodynamic binding parameters for concanavalin A with multivalent sugars at 27 °C

	K_a[a]	$-\Delta G$[b]	$-\Delta H$[c]	$-T\Delta S$[d]	n[e]
	$M^{-1} \times 10^{-4}$		*kcal/mol*		*no. sites/monomer*
Me-αMan[f]	1.2	5.6	8.4	2.8	1.0
1	2.2	6.0	12.7	6.7	0.59
2	2.5	6.0	11.4	5.4	0.67
3	5.3	6.5	15.2	8.7	0.54
p-APMan[g]	1.3	5.6	7.8	2.2	1.0
4	4.7	6.4	17.0	10.6	0.52
5	5.4	6.5	16.6	10.1	0.52
6	6.8	6.6	14.3	7.7	0.60
TriMan[h]	39	7.6	14.7	7.1	1.0
7	286	8.8	23.1	14.3	0.53
8	250	8.7	26.2	17.5	0.53
9	420	9.0	29.0	20.0	0.51
10	1350	9.7	53.0	43.3	0.26

[a] Errors in K_a range from 1 to 7%.
[b] Errors in ΔG are less than 1%.
[c] Errors in ΔH are 1–4%.
[d] Errors in $T\Delta S$ are 1–7%.
[e] Errors in n are less than 2%.
[f] Methyl-α-D-mannopyranoside.
[g] *p*-Aminophenyl-α-D-mannopyranoside.
[h] Methyl-3,6-di-*O*-(α-D-mannopyranosyl)-α-D-mannopyranoside.

consistent with the reported ~40-fold lower affinity of Me-αMan for ConA relative to that of Tri-Man (8), and the nearly 250-fold lower affinity of Me-αMan for DGL relative to that of the trimannoside (19). These differences are due to the extended binding sites of both lectins for the trimannoside as shown in their x-ray crystal structures (20, 21). Thus, analogs

TABLE II
Thermodynamic binding parameters for Dioclea grandiflora lectin with multivalent sugars at 27 °C

	K_a[a]	$-\Delta G$[b]	$-\Delta H$[c]	$-T\Delta S$[d]	n[e]
	$M^{-1} \times 10^{-4}$		*kcal/mol*		*No. sites/monomer*
Me-αMan[f]	0.46	4.9	8.2	3.3	1.0
1	2.0	5.9	11.2	5.3	0.61
2	1.6	5.7	11.0	5.3	0.70
3	10.6	6.8	14.8	8.0	0.56
p-APMan[g]	0.7	5.2	7.3	2.1	1.0
4	1.6	5.7	14.3	8.6	0.60
5	2.5	6.0	14.8	8.8	0.57
6	3.7	6.2	12.0	5.8	0.70
TriMan[h]	122	8.3	16.2	7.9	1.0
7	600	9.3	24.7	15.4	0.50
8	590	9.2	27.5	18.3	0.51
9	1000	9.6	32.2	22.6	0.40
10	6500	10.6	58.7	48.1	0.25

[a] Errors in K_a are 7–10%.
[b] Errors in ΔG are 1%.
[c] Errors in ΔH are 1–7%.
[d] Errors in $T\Delta S$ are 5–13% for analogs **1–6** and 1–2% for analogs **7–10**.
[e] Errors in n are 2–7% for analogs **1–6** and less than 1% for analogs **7–10**.
[f] Methyl-α-D-mannopyranoside.
[g] *p*-Aminophenyl-α-D-mannopyranoside.
[h] Methyl-3,6-di-*O*-(α-D-mannopyranosyl)-α-D-mannopyranoside.

of the trimannoside (**7**-**10**) have intrinsically higher affinities for the two lectins relative to analogs of α-D-mannopyranoside (**1**-**6**).

Analogs **1**-**3** have 2–4-fold higher K_a values for ConA relative to Me-αMan (Table I), and 4–20-fold higher affinities for DGL (Table II). Importantly, the n values for **1**-**3** binding to ConA are considerably lower than 1.0, with values of 0.59, 0.67, and 0.54, respectively. The n values for **1**-**3** binding to DGL are 0.61, 0.70, and 0.56, respectively. The lowest n values are for **3** which possesses the highest K_a values of the three analogs for each lectin. Since the theoretical value of n for divalent binding of a carbohydrate to ConA is $n = 1.0/2 = 0.5$, the n values for **3** of 0.54 and 0.56 for ConA and DGL indicate that it predominately exists in divalent cross-linked complexes with each lectin. The somewhat higher values of n for **1** and **2** indicate a lower percentage of cross-linked molecules in solution relative to that for **3**. Thus, the flexible hexyl chain in **3** appears to favor slightly higher affinity binding and the formation of a higher percentage of cross-linked complexes of the analog with the two lectins, relative to the more rigid acetylenic spacer groups in **1** and **2**.

Analogs **4**-**6** show 4–5-fold higher K_a values for ConA (Table I) relative to Me-αMan, and 2–5-fold higher K_a values for DGL (Table II) relative to Me-αMan. The n values for **4**-**6** binding to ConA are also lower than 1.0, with values of 0.52, 0.52, and 0.60, respectively. The n values for **4**-**6** binding to DGL are 0.60, 0.57, and 0.70, respectively. The somewhat higher n values for DGL relative to ConA appear to correlate with the somewhat higher K_a values of the analogs for ConA relative to DGL. For both lectins the highest K_a values are for **6** which possesses the longest spacer group in this series. However, the lowest n values for ConA are for **4** (0.52) and **5** (0.52), while the lowest n value for DGL is for **5** (0.57). These results indicate that **4** and **5** exists predominately as divalent cross-linked complexes with ConA, and that **5** exists as the highest percentage of cross-linked complex with DGL among the three analogs. Hence, the shorter chain analogs, **4** and **5,** yielded the highest percentage of cross-linked complexes with ConA. Analogs **4** and **5,** with nearly the same n values for DGL, also gave the highest percentages of cross-linked complexes.

The ITC data in Table I for analogs **7** and **8** show 7- and 6-fold higher K_a values for ConA, respectively, relative to monovalent Tri-Man. Both analogs show 5-fold greater K_a values for DGL (Table II) relative to Tri-Man. Hence, the absence or presence of the methyl ester on the benzoic acid moiety of the central phenyl ring has little effect on the affinity of these two analogs. The n values for **7** and **8** binding to ConA are 0.53 for both analogs, and for binding to DGL are 0.50 and 0.51, respectively. These values are close to the theoretical value of 0.50 for binding of a divalent ligand to the two lectins. This indicates that **7** and **8** are primarily involved in divalent cross-linking interactions with the lectins.

Analogs **9** and **10** show 11- and 35-fold higher K_a values for ConA, respectively, and 8- and 53-fold higher K_a values for DGL relative to the trimannoside. Thus, ConA and DGL exhibit substantially higher affinities for tetraantennary analog **10** relative to the corresponding monovalent ligand, Tri-Man. Importantly, n values for **10** binding to ConA and DGL are 0.26 and 0.25, respectively. These values are consistent with the theoretical value of a tetravalent carbohydrate binding to either lectin which is $n = 1.0/4 = 0.25$. This indicates that all four trimannoside moieties of **10** bind to ConA and DGL. The fact that both lectins precipitate with **10** after the ITC experiments suggests that each molecule of **10** cross-links four lectin molecules, respectively, and forms a two- or three-dimensional cross-linked complex which is generally required for precipitation of the complex (12).

*The Functional Valency of **9** Differs from Its Structural Valency for ConA and DGL*—The above results show that the values of n for binding of **7, 8,** and **10** to ConA and DGL are inversely proportional to the number of binding epitopes in the analogs. However, the value of n for the binding of **9** to ConA is 0.51 instead of the predicted value of 0.33 based on the structural valency of the triantennary analog which posessess three trimannoside residues. Thus, **9** is functionally bivalent in binding to ConA as indicated by its ITC derived value of n. On the other hand, the n value for **9** binding to DGL is 0.40 which is less than 0.50 for divalent binding but higher than 0.33 for trivalent binding. The n value of 0.40 suggest that greater than half of the molecules of **9** are involved in trivalent binding to DGL and less than half are involved in bivalent binding to the lectin. Thus, the ITC results show that analog **9** is divalent in binding to ConA, and divalent and trivalent in binding to DGL. The reason for this difference in functional valency of **9** for the two lectins is unknown, but there has been a report of differences in the binding of divalent C-glycosides to ConA and DGL (34). This difference in functional valency of **9** for ConA and DGL is also interesting in light of the similarity in overall structures of the two lectins (21). The results for **9** also indicate the importance of ITC measurements in determining the *functional* valency of a multivalent carbohydrate for specific lectins, which may differ from the structural valence of the carbohydrate.

ΔH Increases in Direct Proportion to the Valency of High Affinity Carbohydrate Analogs—The present study indicates that multivalent analogs with relatively high affinities possess n values for ConA and DGL that reflect the functional and, most often, nominal structural valency of the ligands. Present results also show that for higher affinity multivalent analogs, the observed value of ΔH per mole of the analog is approximately the sum of the ΔH values of the individual epitopes. Similar observations have been made for the binding of a trivalent system of receptor and ligand derived from vancomycin and D-Ala-D-Ala (37). Examples in the present study include divalent analogs **4** and **5** binding to ConA ($n = 0.52$ for both) (Table I), which have ΔH values of -17.0 kcal mol^{-1} and -16.6 kcal mol^{-1}, respectively. Assuming that p-aminophenyl-α-D-mannopyranoside approximates the binding thermodynamics of the monovalent epitopes in **4** and **5,** the ΔH value of p-aminophenyl-α-D-mannopyranoside is -7.8 kcal/mol which is nearly half that of **4** and **5** (Table I).

Divalent analog **3** possesses an n value of 0.54 for binding to ConA and a ΔH of -15.2 kcal/mol. Me-αMan ($n = 1.0$) as the corresponding monovalent epitope binding to ConA has a ΔH of -8.4 kcal/mol, which is nearly half that of **3**. Analogs **1** and **2** with higher n values than that of **3** show ΔH values between that of Me-αMan and **3,** indicative of partial divalent binding of the former two analogs to ConA.

Divalent analogs **7** ($n = 0.53$ to ConA, and 0.50 for DGL) and **8** ($n = 0.53$ to ConA, and 0.51 for DGL) are also relatively high affinity divalent carbohydrates. **7** and **8** have ΔH values of -23.1 and -26.2 kcal/mol, respectively, for binding to ConA which are less than twice the ΔH value of -14.7 kcal/mol for Tri-Man (Table I). The same is true for **7** and **8** binding to DGL (Table II). The ΔH value for tetravalent analog **10** binding to ConA is -53.0 kcal/mol, which is approximately four times as great as the ΔH value of the free trimannoside (-14.7 kcal/mol). The ΔH value for **10** binding to DGL is -58.7 kcal/mol, which is also nearly four times as great as the ΔH value of the free trimannoside (-16.2 kcal/mol). The ΔH values of analog **9** are more complicated since the analog appears to be close to bivalent for ConA ($n = 0.51$), and partially trivalent for DGL ($n = 0.40$). The above results, therefore, provide evidence that the ΔH values of relatively high affinity multivalent carbohydrates binding to ConA and DGL are approximately the sum of the ΔH values of the individual binding epitopes in the analogs.

$T\Delta S$ Does Not Directly Increase in Proportion to the Valency of High Affinity Carbohydrate Analogs—The K_a values for analogs **1**-**10** binding to ConA and DGL in Tables I and II, respectively, are greater than those of the corresponding monovalent carbohydrates. The largest increases in K_a values are for tetravalent analog **10** binding to ConA (35-fold) and DGL (53-fold). However, these enhancements are small compared with the very large increases in binding that occur when a multivalent ligand binds to a single molecule possessing multivalent binding sites. Examples of the latter are binding of a triantennary complex carbohydrate to the hepatic asialoglycoprotein receptor which has a $\sim 10^{-9}$ M inhibition constant relative to the $\sim 10^{-3}$ M inhibition constant of the corresponding monovalent oligosaccharide (15). Even more dramatic is the increase in affinity to $\sim 10^{17}$ M^{-1} of a trivalent derivative of vancomycin binding to a trivalent derivative of D-Ala-D-Ala in which the affinity of the corresponding monovalent analogs is $\sim 10^{6}$ M^{-1} (37). In the latter study, thermodynamic measurements showed that both ΔH and $T\Delta S$ scaled proportionally to the number of binding epitopes in the molecules. These thermodynamic findings are characteristics of the binding of a multivalent ligand to a single multivalent receptor molecule.

However, in the present study, ΔH scales proportionally to the number of binding epitopes in the higher affinity multivalent carbohydrates in Tables I and II (compare **7, 8** and **10**), but $T\Delta S$ does not. Instead, $T\Delta S$ is much more negative than if it proportionally scaled to the number epitopes in the carbohydrates. For example, **10** contains four trimannosyl binding epitopes, and to a first approximation, the ΔH value for ConA of -53 kcal/mol is four times the ΔH of -14.7 kcal/mol for Tri-Man (Table I). However, the observed TΔS value for **10** is -43.3 kcal/mol, not -28.4 kcal/mol if it scaled with the $T\Delta S$ value of -7.1 kcal/mol for Tri-Man (Table I). The resulting ΔG value of **10** would also be much greater if $T\Delta S$ scaled with valency since the difference between ΔH and $T\Delta S$ would be greater. However, the observed ΔG value(s) for **10** are much smaller. The

same is true for the other multivalent carbohydrates in Tables I and II. Hence, the finding in the present study that ΔH values scale but not $T\Delta S$ values is characteristic of the binding of a multivalent ligand to separate, unconnected receptor molecules. In the present case, binding of a single molecule of **10** to four separate ConA or DGL molecules.

Relative Contributions of ΔH and $T\Delta S$ to the Increased Affinities of Multivalent Carbohydrates ***1–10***—The enhancements in affinities of **1**-**10** to ConA and DGL, relative to their monovalent analogs, are not due to their binding to a single tetrameric lectin molecule in each case (above results). For example, the enhancements in affinity of **10** for ConA (35-fold) and DGL (53-fold) relative to Tri-Man are not due to **10** binding to single molecules of ConA or DGL that possess four binding sites. Rather, the increases in affinity of **10** are due to binding of four separate lectin molecules to **10**. This means that the observed K_a value for **10** is actually the average of the four microscopic K_a values at each of its four epitopes, since each epitope is involved in binding to a separate lectin molecule. It therefore follows that if ΔH is constant at each epitope, as determined above, and is approximately the same as that of Tri-Man, then increases in the overall microscopic K_a values at the four epitopes require more favorable $T\Delta S$ contributions than that of Tri-Man. A detailed analysis of these microscopic equilibria parameters will be presented elsewhere.[2] However, an estimate of the favorable macroscopic $T\Delta S$ contribution to the enhanced affinity of **10** relative to Tri-Man can be obtained by considering that the differences in ΔG values between **10** and Tri-Man for the two lectins is the enhancement in $T\Delta S$ values. The difference in ΔG values for ConA and DGL binding **10** are 2.1 and 2.3 kcal/mol, respectively, which can be considered as the favorable contribution to binding of $T\Delta S$ for **10** relative to Tri-Man to the two lectins.

In terms of a physical model for the enhanced affinities of the multivalent carbohydrates in Tables I and II, increased favorable $T\Delta S$ contributions for binding of the analogs can be analyzed in kinetic terms. Since $K_a = k_1/k_{-1}$, the ratio of the forward and reverse rate constants for binding, the enhanced affinity constants of the multivalent analogs can be due to either an increase in k_1 or a decrease in k_{-1}, or both. Since each analog has microscopic K_a values at each epitope, it follows that an increase in the corresponding microscopic rate constant k_1 or decrease in the microscopic off-rate, k_{-1}, will enhance binding at each epitope and hence the overall macroscopic K_a value. These changes in microscopic rate constants associated with the more favorable $T\Delta S$ constributions at each epitope are associated with physically "clustering" binding epitopes in a molecule. More details of this phenomena will be presented elsewhere.

Entropy-Enthalpy Compensation Plots—Enthalpy-entropy plots ($-\Delta H$ versus $-T\Delta S$) of the data in Tables I and II for analogs **1–10** are shown in Fig. 6*A*. Remarkably, straight line fits of the data for both lectins are observed with slopes of 1.11 for ConA and 1.13 for DGL (correlation coefficients of 0.99 for both). For comparison, Fig. 6*B* shows enthalpy-entropy plots for binding of a series of monovalent carbohydrates to ConA and DGL which are listed in the figure legend. All of the monovalent sugars used in the two plots in Fig. 6*B* bind to ConA and DGL with n values close to 1.0 (8, 38). The slopes of their $-\Delta H$ *versus* $-T\Delta S$ plots are 1.52 for ConA and 1.55 for DGL, with correlation coefficients of 0.98 and 0.99, respectively. The plots for both lectins are similar as previously noted (8, 38). The slopes for **1**-**10** binding to ConA (1.11) and DGL (1.13) are less than those for the monovalent carbohydrates because the magnitude of the ΔH and $T\Delta S$ values for **1**-**10** are larger relative to their ΔG values as compared with the monovalent sugars which possess smaller ΔH and $T\Delta S$ values relative to their ΔG values.

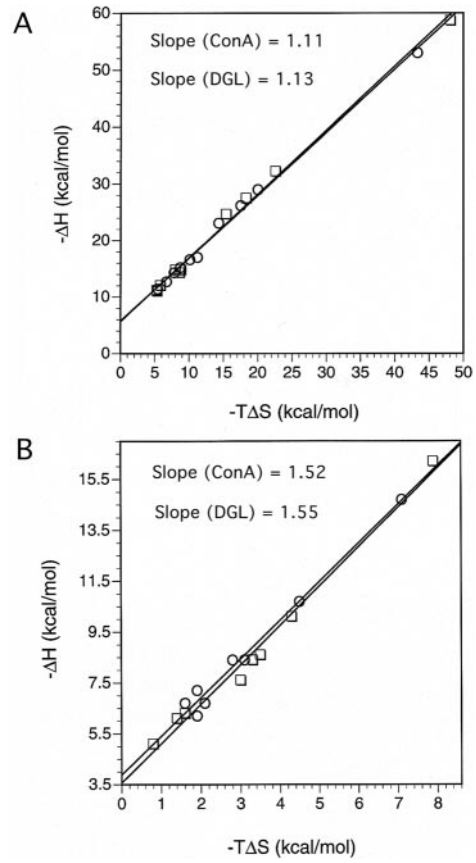


FIG. 6. **Plots of $-\Delta H$ *versus* $-T\Delta S$ for the binding of: *A*, analogs 1–10 to ConA (○) and DGL (□); and *B*, Me-α-D-glucopyranoside, methyl-α-2-deoxy-α-D-glucopyranoside, maltose, isomaltose, Manα(1–3)Man, Manα(1–6)Man, and Tri-Man to ConA (○) and DGL (□), and at 27 °C.** The correlation coefficient for the fit of the data in *A* for ConA and DGL were 0.98 and 0.99, respectively. The correlation coefficient for the fit of the data in *B* for ConA and DGL were 0.99 for both. The resulting slopes from the fits are given in *A* and *B*. Thermodynamic values were obtained from Refs. 8 and 38 and the present study.

Summary—The present ITC study indicates that the value of n, the number of binding sites on each monomer of ConA or DGL, is inversely proportional to the number of binding epitopes in relatively high affinity multivalent carbohydrates. The value of n for these carbohydrates can therefore determine the functional valency of a sugar as opposed to its structural valency, as observed for binding of **9** to ConA and DGL. The observed ΔH values of relatively high affinity multivalent carbohydrates are approximately the sum of the ΔH values of the individual epitopes in the molecules. Evidence indicates that the greater K_a values of multivalent carbohydrate **1**-**10** to ConA and DGL, relative to monovalent analogs, are due to more positive contributions in their $T\Delta S$ terms. A thermodynamic model for distinguishing binding of a multivalent ligand to a single receptor with multiple, equal subsites (37) *versus* binding of a multivalent ligand to separate receptor molecules is presented.

REFERENCES

1. Varki, A. (1993) *Glycobiology* **3,** 97–101
2. Lis, H., and Sharon, N. (1998) *Chem. Rev.* **98,** 637–674
3. Witczak, Z. J. (1997) in *Carbohydrates in Drug Design* (Witczak, Z. J., and Nieforth, K. A., eds) pp. 1–38, Marcel Dekker, Inc., New York
4. Wong, C.-H. (1999) *Acc. Chem. Res.* **32,** 376–385
5. Quiocho, F.-A. (1989) *Pure Appl. Chem.* **61,** 1293–1299
6. Toone, E. J. (1994) *Curr. Opin. Struct. Biol.* **4,** 719–728
7. Lemieux, R. U. (1996) *Acc. Chem. Res.* **29,** 373–380

8. Mandal, D. K., Kishore, N., and Brewer, C. F. (1994) *Biochemistry* **33,** 1149–1156
9. Lee, Y. C. (1993) *FASEB J.* **6,** 3193–3200
10. Roy, R. (1996) *Curr. Opin. Struct. Biol.* **6,** 692–702
11. Kiessling, L. L., and Pohl, N. (1996) *Chem. Biol.* **3,** 71–77
12. Brewer, C. F. (1996) *Chemtracts Biochem. Mol. Biol.* **6,** 165–179
13. Olsen, L. R., Dessen, A., Gupta, D., Sabesan, S., Sacchettini, J. C., and Brewer, C. F. (1997) *Biochemistry* **36,** 15073–15080
14. Drickamer, K. (1995) *Nat. Struct. Biol.* **2,** 437–439
15. Lee, Y. C., Townsend, R. R., Hardy, M. R., Lonngren, J., Arnarp, J., Haraldsson, M., and Lonn, H. (1983) *J. Biol. Chem.* **258,** 199–202
16. Zanini, D., and Roy, R. (1997) *Bioconjug. Chem.* **8,** 187–192
17. Chervenak, M. C., and Toone, E. J. (1995) *Biochemistry* **34,** 5685–5695
18. Chervenak, M. C., and Toone, E. J. (1996) *Bioorg. Med. Chem.* **4,** 1963–1977
19. Dam, T. K., Oscarson, S., and Brewer, C. F. (1998) *J. Biol. Chem.* **273,** 32812–32817
20. Naismith, J. H., and Field, R. A. (1996) *J. Biol. Chem.* **271,** 972–976
21. Rozwarski, D. A., Swami, B. M., Brewer, C. F., and Sacchettini, J. C. (1998) *J. Biol. Chem.* **273,** 32818–32825
22. Moreira, R. A., Barros, A. C. H., Stewart, J. C., and Pusztai, A. (1983) *Planta* **158,** 63–69
23. Agrawal, B. B. L., and Goldstein, I. J. (1967) *Biochim. Biophys. Acta* **147,** 262–271
24. Goldstein, I. J., and Poretz, R. D. (1986) in *The Lectins* (Liener, I. E., Sharon, N., and Goldstein, I. J., eds) pp. 35–244, Academic Press Inc., New York
25. Roy, R., Das, S. K., Donimique, R., Trono, M. C., Mateo, F. H., and Gonzalez, F. S. (1999) *Pure Appl. Chem.* **71,** 565–571
26. Page, D., and Roy, R. (1997) *Glycoconj. J.* **14,** 345–356
27. Roy, R., Page, D., Perez, S. F., and Bencomo, V. V. (1998) *Glycoconj. J.* **15,** 251–263
28. Saha, S. K., and Brewer, C. F. (1994) *Carbohydr. Res.* **254,** 157–167
29. Wiseman, T., Williston, S., Brandt, J. F., and Lin, L.-N. (1989) *Anal. Biochem.* **179,** 131–135
30. Naismith, J. H., Emmerich, C., Habash, J., Harrop, S. J., Helliwell, J. R., Hunter, W. N., Raftery, J., Kalb-Gilboa, A. J., and Yariv, J. (1994) *Acta Crystallogr. Sect. D* **50,** 847–858
31. Gunther, G. R., Wang, J. L., Yahara, I., Cunningham, B. A., and Edelman, G. M. (1973) *Proc. Natl. Acad. Sci. U. S. A.* **70,** 1012–1016
32. Bhattacharyya, L., and Brewer, C. F. (1989) *Eur. J. Biochem.* **178,** 721–726
33. Gupta, D., Oscarson, S., Raju, T. S., Stanley, P., Toone, E. J., and Brewer, C. F. (1996) *Eur. J. Biochem.* **242,** 320–326
34. Weatherman, R. V., Mortell, K. H., Chervenak, M., Kiessling, L. L., and Toone, E. J. (1996) *Biochemistry* **35,** 3619–3624
35. McKenzie, G. H., Sawyer, W. H., and Nichol, L. W. (1972) *Biochim. Biophys. Acta* **263,** 283–293
36. Huet, M. (1975) *Eur. J. Biochem.* **59,** 627–632
37. Rao, J., Lahiri, J., Isaacs, L., Weis, R. M., and Whitesides, G. M. (1998) *Science* **280,** 708–711
38. Dam, T. K., Oscarson, S., Sacchettini, J. C., and Brewer, C. F. (1998) *J. Biol. Chem.* **273,** 32826–32832

Binding of Multivalent Carbohydrates to Concanavalin A and*Dioclea grandiflora* Lectin: THERMODYNAMIC ANALYSIS OF THE ''MULTIVALENCY EFFECT''

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