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Some physicochemical aspects of oligosaccharide binding to concanavalin A and wheat germ agglutinin

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Abstract. The binding of fluorescently labelled carbohydrates to concanavalin A and wheat germ agglutinin was studied at equilibrium and by the stopped-flow and temperature jump relaxation methods. Ligand were mainly die 4-methylumbelliferyl glycosides of α (1 → 2)-linked manno-oligosaccharides and of β (1 → 4)-linked chito oligosaccharides as limited homologous series. They offer distinct advantages, parti cularly for kinetic studies.

Enthalpie and kinetic considerations suggest that concanavalin A specifically binds a single mannopyranosyl group in α (1→2)-linked manno-oligosaccharides. This occurs preferentially at the non-reducing end. Glycosylation of a carbohydrate with *e.g.* an aryl group does not afect die binding kinetics and for all carbohydrates the association rate is comparable but relatively slow, which indicates that a common process is involved in the binding of all carbohydrates to concanavalin A. The affinity of a carbohydrate for concanavalin A is determined by the dissociation-rate parameter, resulting in a longer residence time for a better ligand.

Interaction of chito-oligosaccharides with wheat germ agglutinin is complex. With the larger members of the 4-methylumbelliferyl chito-oligosaccharides, binding studies were only possible at low fractional saturation to avoid formation of unsoluble complexes. The binding kinetics of wheat germ agglutinin are faster than with concanavalin A and are consistent with a wheat germ agglutinin binding region composed of two adjacent subsites. For binding of the monoside as well as the bioside, two consistent kinetic models apply. They have common that for each ligand there exist two complexes with comparable population.

Keywords. Concanavalin A; wheat germ agglutinin; fluorescence; difference absorption; temperature-jump; stopped-flow.

Introduction

In 1973, Dean and Homer reported that the fluorescence of 4-methylumbelliferyl α-D-mannopyranoside (MeUmb-Man*p*) is totally quenched upon binding to concanavalin A (Con A). Since then, MeUmb-glycosides have emerged as interesting ligands for equilibrium and kinetic studies of carbohydrate-protein interactions. Examples are the studies with the lectins Con A (Loontiens *et al.,* 1977a, b; Clegg *et al.,* 1977, 1981; Van Landschoot *et al*.,1978a, 1980a, b; Fitos *et*

Abbreviations used: MeUmb-Man*p*- 4-methylumbelliferyl α-D-mannopyranoside; Con A, concanavalin A; WGA, wheat germ agglutinin; Me-Man*p*, methyl-α-D-mannopyranoside.

al., 1979; Farina and Wilkins, 1980), wheat germ agglutinin (WGA) (Privat *et al.,* 1974b; Van Landschoot *et al.,* 1977, 1978b; Clegg *et al.,* 1980; Erni *et al.,* 1980) and those from the seeds of *Ricinus communis* (Khan *et al.,* 1980), *Momordica charantia* (Khan *et al.,* 1981a), *Abrus precatorius* (Khan *et al.,* 1981b), *Bandeiraea* (*Griffonia*) *simplicifolia* (De Boeck *et al.,* 1981), *Pisum sativum* (De Boeck, 1980), *Erythrina cristagalli* (unpublished; in collaboration with J. Iglesias, H. Lis and N. Sharon). Other systems include GlcNAc-directed antibodies (Kieda *et al.,* 1977), lysozyme (Delmotte *et al.,* 1975; Yang and Hamaguchi, 1980a, b) and a β-xylosidase from *Bacillus pumilus* (Claeyssens and De Bruyne, 1978).

This interest in MeUmb-glycosides is due to their versatility in such binding studies. Their large absorption coefficient (about 1.3×10^4 M^{-1} cm^{-1} at 316 to 318 nm) and their fluorescence (maximal emission at 373 nm) allow very sensitive detection. Upon binding to a protein, these ligands often show pronounced difference absorption spectra (in the 300 to 350 nm range) as well as changes in ligand fluorescence properties. In many cases there is a drastic or total quenching of the fluorescence intensity (with lysozyme, an increase is observed). When no quenching occurs, or when it is incomplete, binding can be followed by the substantial increase in ligand fluorescence polarisation (Van Landschoot *et al*.,1978a; Khan *et al.,* 1980a, b; De Boeck *et al.,* 1981; unpublished results with *Erythrina cristagalli*). All these spectroscopic changes are intense, and were found to be specifically inhibited by the appropriate carbohydrates. Such MeUmb derivatives are particularly valuable for binding studies where nonchromophoric carbohydrates cause little, if any, change in the intrinsic optical properties of a carbohydrate-binding protein such as *e.g.* Con A. In addition, MeUmb-glycosides have been used to study the binding equilibrium (Dean and Homer, 1973; Van Landschoot *et al.,* 1980a) and kinetics (Clegg *et al.,* 1981) of nonchromophoric carbohydrates to Con A. Similarly, MeUmb-glycosides are sensitive indicators of the reactivation of the lectins when the appropriate metal ions are added to a demetallized lectin such as Con A (Harrington and Wilkins, 1978; Christie *et al.,* 1978, 1979; Urdea *et al.* , 1979; De Clercq *et al.,* 1980) or to a mixture of demetallized isolectins I from *Bandeiraea* (*Griffonia*) *simplicifolia* (Harrington *et al.,* 1981).

Here we summarize our results on the binding equilibria and kinetics of α (1 → 2)-linked manno-oligosaccharides [(Man*p*)$_n$] with Con A, and of chito-oligosaccharides, [(GlcNac*p*)$_n$] with WGA. In addition to these ligands, we have mainly used the MeUmb (Man*p*)$_n$ α-glycosides and MeUmb (GlcNac*p*)$_n$ β-glycosides with n = 1, 2 or 3. For the latter lectin-bound glycosides, the sign or in intensity of their difference absorption spectra, as well as the degree of ligand-fluorescence quenching, reflect the average surrounding of the MeUmb group on these lectins. The binding characteristics of the parent carbohydrates were determined from substitution titrations with a MeUmb-glycoside as a fluorescent indicator ligand (Van Landschoot *et al.,* 1980a). The binding kinetics of the MeUmb-glycosides were studied by the temperature jump relaxation technique (Clegg *et al.,* 1977, 1980; Loontiens *et al.,* 1977b; Van Landschoot *et al.,* 1980b) and stopped-flow method (Clegg *et al.,* 1977; Van Landschoot *et al.,* 1980b), for methyl α-D-mannopyranoside (Me-Man*p*) a competitive temperature-jump relaxation method was used (Clegg *et al.,* 1981).

Results

Con A[⋆]

Equilibrium studies with Con A: The equilibrium binding experiments have provided information on the affinity and on the spectroscopic changes which are involved in Con A-carbohydrate interactions. Low molecular weight carbohydrates like MeUmb-Man*p* and MeUmb $(Man p)_2$ bind to one site per protomer on Con A, and do so in an independent fashion, both in its dimeric and tetrameric forms. This was shown by equilibrium dialysis, absorption difference spectra titrations (Loontiens *et al.,* 1977a; Van Landschoot *et al.,* 1978a, 1980b) and is very apparent from the Scatchard plot in figure 1, which is obtained from titrations using the total fluorescence quenching of MeUmb-Man*p* when bound to Con A. Interaction of the lectin with these simple carbohydrate derivatives in solution does not show any of the complex binding phenomena which have been reported for membrane bound cell-wall receptors. These have been suggested (Wright, 1980b) to be due to the aggregation of Con A (and WGA) at the cell surface which is dependent upon the concentration of the lectin as well as the carbohydrate. A summary of the binding characteristics for $(Man p)_n$ and MeUmb $(Man p)_n$ is listed in table 1 (Van Landschoot *et al.,* 1978a). The preliminary assignment of the binding configuretion shown with table 1 is suggested by the optical-signal changes. The value of the change in enthalpy (Δ H°) for all ligands with α (1 $\rightarrow$ 2) mannosidic linkages is independent of the number of Man*p* groups, or of the presence of a MeUmb group. Since hydrogen bonds, involved in the binding of carbohydrates have a much larger contribution in Δ H than nonspecific interactions, this would imply that Con A specifically binds a single Man*p* group in α (1 $\rightarrow$ 2)-linked manno-oligosaccharides[⋆⋆]. The value of ΔH° for Me-Man*p* in table 1 has been confirmed by calorimetric measurements at comparable ionic strength (Munske *et al*.,1978). The close relation between Δ H° and the carbohydrate-binding affinity is illustrated by comparing the poorer ligand Me α-Glc*p* (Δ H° = – 18 kJmol^{-1})[†] with Me-Man*p* (Δ H° = – 38 kJmol^{-1}) (Clegg *et al.,* 1981).

Kinetic studies with Con A: All of the equilibrium experiments have been interpreted assuming a single binding configuration between the carbohydrates and the protein. This is probably not valid for the oligosaccharides containing more than one Man*p* group. Furthermore, from the equilibrium binding studies it is difficult to obtain information whether the carbohydrate binding site in each protomer can simultaneously interact with more than one Man*p* group. On the contrary, the

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⋆ All equilibrium and kinetic data for binding of carbohydrates and Con A were obtained with the fully metellized lectin (Ni^{2+}, Ca^{2+}) that was virtually free of nicked polypeptide chains. The conditions were: 1 M NaCl at pH 5.5 (dimer) or at pH 7.2 (tetramer) in a temperature range of 4 to 40°C. All results are expressed on the basis of Con A protomer with M_r equal to 25,500.

⋆⋆ The conclusion for these ligands can result from steric hindrance that does not occur upon binding of oligosaccharides derived from N-glycopeptides (J. Carver, communicated).

† These values were obtained by simulating and concentration dependence of the Δ H-sensitive amplitudes in competition temperature-jump relaxations with MeUmb-Man*p* as a fluorescent kinetic indicator (Clegg *et al.,* 1981). The ratio of these values for the two Me-glycosides was confirmed by calorimetry under different conditions of pH and at low ionic strength (Dani *et al.,* 1981).

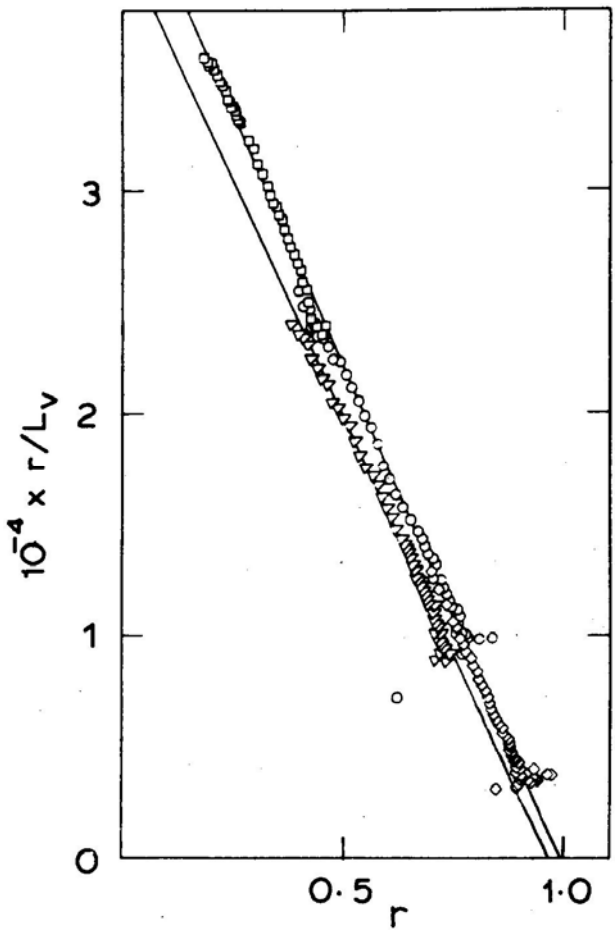

Figure 1. Scatchard plots for the binding of MeUmb-Man*p* to Con A at pH 5.5 and 15.8°C (De Boeck, 1980). The right-hand graph is for linearized titrations acquired by observing the fluorescence quenching of MeUmb-Man*p* during the addition of native Con A to 554 µl of 20.6 µM (□), 87.4 µM (O) and 275 µM (◇) MeUmb-Man*p* contained in a thermostated 0.5×0.5×4.5-cm cuvette equipped with a motor driven stirrer; 0 to 200 µl of 793 µM native Con A was added from a microburette. Excitation of fluorescence was in the 335 to 358 nm range, with absorbance < 0.02 and emission was measured at 373 to 380 nm. All titration data were corrected for a blank titration without MeUmb-Man*p*) as well as for dilution; they correspond to K = (4.51 ± 0.02) 10_4 M^{-1} with 0.985 ± 0.005 binding sites per protomer. The data of the left-hand graph were obtained from a single titration experiment with 87.4 µM MeUmb-Man*p* and a sample of Con A (606 µM) that had first been demetallized, and then reactivated with an excess of Ni^{2+} and Ca^{2+}. These plots show that the binding properties of native, and of reactivated Con A are practically identical.

above data do not indicate the existence of an extended binding region composed of several subsites. By extending the studies into the time domain, we have obtained information about multiple binding possibilities. The following experiments with α (1 → 2)-linked manno-oligosaccharide derivatives are consistent with a single binding site per protomer of Con A. It interacts with only one Man*p* group at a time, but it can interact differently with any, out of several Man*p* groups in these oligosaccharides (see scheme II of table 2).

Table 1. Characteristics for the binding of α (1→2)-linked MeUmb (Man*p*)$_n$, (Man*p*)$_n$ and Me-Man*p* to Con A at 25°C.

	pH	$10^3 \times \Delta\varepsilon_{334}$ ($M^{-1}\,cm^{-1}$)	F_∞/F_0	$10^{-4} \times K$ (M^{-1})	$-\Delta H°$ ($kJmol^{-1}$)	$-\Delta S°$ ($Jmol^{-1}\,K^{-1}$)
MeUmb-Man*p*	5.5	−2.21	0.00±0.02	3.36±0.04	34.8±0.4	30± 1
MeUmb-Man*p*	7.2	−2.21	0.01±0.02	4.26±0.07	35.6±0.8	31± 3
MeUmb-(Man*p*)$_2$	7.2	+ 1.04	0.34±0.01	13.9 ±0.5	36.0±0.8	22± 3
MeUmb (Man*p*)$_3$	7.2	+ 0.44	0.40±0.01	8.2 ±0.3	36.8±0.8	29± 4
Me-Man*p*	5.5	–	–	0.64±0.05	38 ±2	56± 7
(Man*p*)$_2$	5.5	–	–	1.2 ±0.1	33 ±4	32±14
(Man*p*)$_3$	5.5	–		11.0 ±0.5	37 ±2	31± 7

For binding of MeUmb (Man*p*)$_n$, $\Delta\varepsilon_{334}$ is the change in the molecular extinction coefficient at 334 nm. F_∞/F_0 is the fluorescence of the complex relative to the fluorescence of its free ligand. The data for MeUmb (Man*p*)$_n$ were obtained by fluorescence titrations at six to ten temperatures and by difference absorption measurements at 334 nm; they are consistent with the results obtained by equilibrium dialysis (Loontiens *et al.*, 1977a; Van Landschoot *et al.*, 1978a, 1980b). The data for the nonchromophoric compounds were obtained from substitution titrations with MeUmb-Man*p* as a fluorescent indicator ligand; the corresponding Scatchard plots were linear provided that a slowly establishing equilibrium, *e.g.* with (Man*p*)$_2$, was taken into account (Van Landschoot *et al.*, 1980a).

Preliminary binding scheme:

```
      A    X    Y
      M  –  F
      M  –  M  –  F
M  –  M  –  M  –  F
     (M  –  M  –  M  –  F)
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(Scheme I)

X and Y represent distances from site A with M = Man*p* and F = MeUmb. This scheme will be refined into scheme (II) (table 2) in view of the kinetic results.

Table 2. Reaction-rate parameters and signal changes per complex for binding of MeUmb (Man*p*)$_2$ to Con A at 20°C and pH 5.5 according to two consistent mechanisms.

Sliding (sequential)		Alternating (excluded site)	
$A+B \underset{-1}{\overset{+1}{\rightleftharpoons}} C_1 \underset{-2}{\overset{+2}{\rightleftharpoons}} C_2$		$C_a \underset{-a}{\overset{+a}{\rightleftharpoons}} A+B \underset{-b}{\overset{+b}{\rightleftharpoons}} C_a$	
k_{+1}	$6.4\times10^4\ M^{-1}\ sec^{-1}$	k_{+a}	$3.6\times10^4\ M^{-1}\ sec^{-1}$
k_{-1}	$5.7\ sec^{-1}$	k_{+a}	$10.1\ sec^{-a}$
k_{+2}	$4.3\ sec^{-1}$	k_{+b}	$2.8\times10^4\ M^{-1}\ sec^{-1}$
k_{-2}	$0.2\ sec^{-1}$	k_{-b}	$0.12\ sec^{-1}$
K_1	$k_{+1}/k_{-1}=1.1\times10^4\ M^{-1}$	K_a	$k_{+a}/k_{-a}=3.5\times10^3\ M^{-1}$
K_2	$k_{+2}/k_{-2}=21$	K_b	$k_b/k_{-b}=2.4\times10^5\ M^{-1}$
K_I	$K_1(1+K_2)=2.5\times10^5 M^{-1}$	K_{II}	$K_a+K_b=2.5\times10^5\ M^{-1}$
$F_\infty/F_0(C_1)$	0.33	$F_\infty/F_0(C_a)$	0.31
$F_\infty/F_0(C_2)$	0.35	$F_\infty/F_0(C_b)$	0.35
$\Delta\varepsilon_1(C_1)$	$570\ M^{-1}\ cm^{-1}$	$\Delta\varepsilon_1(C_a)$	$210\ M^{-1}\ cm^{-1}$
$\Delta\varepsilon_2(C_2)$	$1070\ M^{-1}\ cm^{-1}$	$\Delta\varepsilon_2(C_b)$	$1040\ M^{-1}cm^{-1}$

The data were obtained by stopped-flow kinetics measuring changes in ligand fluorescence [0.1 and 1 μM MeUmb (Man*p*)$_2$] and ligand absorption [21 μM MeUmb (Man*p*)$_2$] using more than twenty different concentrations of Con A (6 to 340 μM) in excess under pseudo first-order conditions (Van Landschoot *et al.,* 1980b). The stopped-flow traces showed a faster apparent reaction-rate constant (110 to 37 m sec) with a saturable amplitude and a slower one (3.9 to 0.3 sec) with a maximal amplitude at 30 μM. The reaction-rate parameters were calculated from linear plots of the sum and of the product of the apparent reaction-rate constants *versus* the sum of the free concentrations of ligand and protein (Bernasconi, 1976). The values for the signal change per complex ($F\infty/F_0$ and Δε, as defined in table 1) differ for each mechanism. The signal changes for C_2 or C_b are almost identical to those at equilibrium (table 1) due to the preferred binding of this complex *via* its terminal non-reducing Man*p* as in scheme (I). The value of the kinetically defined association constant ($2.5\times10^5\ M^{-1}$) is in fair agreement with the equilibrium value ($1.8\times10^5\ M^{-1}$) at 20° C.
Binding scheme:

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      A    X    Y
M  –  M  –  F        Ca or C1
      M  –  M  –  F  Cb or C2
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(Scheme II)

with abbreviations as for scheme (I).

If a monosaccharide were to bind to a lectin with an extended binding region composed of subsites, the number of binding possibilities could equal the number of these subsites. It is therefore relevant that the binding of all monosaccharides that have been investigated with Con A, show a monophasic kinetic process corresponding to a single-step association (Gray and Glew, 1973; Brewer *et al.,* 1973, 1974; Lewis *et al.,* 1976; Clegg *et al.,* 1977, 1981; Loontiens *et al.,* 1977b; Williams *et al.*,1978b, 1981; Farina *et al.*,19 80). This indicates that the binding region contains but one Man*p*-specific subsite. A detailed temperature jump relaxa-

tion study (Clegg *et al.,* 1977; Loontiens *et al.,* 1977b) with MeUmb-Man*p* and dimeric or tetrameric Con A, showed a single relaxation of the ligand fluorescence or absorption which is related to ligand binding. The dependence of the relaxation time τ and amplitude on the concentration of ligand or protein is consistent with a bimolecular one-step association over a wide concentration range (upto 400 μM). The activation energy for association is high (63 $kJmol^{-1}$) and the activation volume is positive (16 ml mol_{-1}) (Fitos *et al.*,1979). Two minor relaxations of protein fluorescence ($\tau \simeq 10$ m sec and $\tau > 1$ sec) were also observed by temperature-jump and pressure-jump (Elson E. L. unpublished) techniques, but it was established (Clegg *et al.,* 1977), that these were unrelated to the binding of carbohydrates, or of metal ions. In addition, the latter two relaxations are unrelated to the dimer-tetramer equilibrium.

Other evidence that a single Man*p* group is involved in the binding is available from the experiments with the fluorescently labelled manno-oligosaccharides. In particular, the complexity of the kinetic progress curves under first-order conditions increases as the number of the Man*p* increases, *i.e.* the stopped-flow progress curves are mono-, bi- or triphasic for n = 1, 2 or 3 of MeUmb $(Manp)_n$ (Van Landschoot *et al.,* 1980b).

The kinetic characteristics for MeUmb $(Manp)_2$ binding to Con A are summa rized in table 2. The concentration dependencies of the apparent rate constants (a linear increase for the faster process, and a hyperbolic increase for the slower process), and of the corresponding amplitudes are consistent with two nearly indistinguishable mechanisms (Viale, 1971; Van Landschoot *et al.,* 1980b). As shown in table 2, each proposed mechanism involves two complexes with different fluorescence and absorption properties depending on the mechanisms. The first mechanism consists of the bimolecular formation of a complex C_1 that subsequently isomerizes into C_2. This can be interpreted as a sliding movement of MeUmb $(Manp)_2$ on the protein surface. For this mechanism we propose that MeUmb $(Manp)_2$ is initially bound as C_1 through the internal Man*p*) at A in scheme (II) and subsequently translocates to form C_2 such that the terminal Man*p* is now bound at A. It is this last step that forms the preferentially bound species at equilibrium. The second mechanism considers the same two bound species (C_1 and C_2 for mechanism I, C_a and C_b for mechanism II), but assumes that a Con A-MeUmb $(Manp)_2$ complex must completely dissociate before binding again in a bimolecular step. The complex C_b is suggested to have its terminal Man*p* bound to site A and, as with scheme (I), this is the more affine complex. However, for scheme (II) the difference in the populations of the two complexes is more pronounced than for scheme (I) (Scheme I, C_2/C_1 = 21; scheme II, C_b/C_a= 68). The second mechanism (alternating) provides the best opportunity to compare the binding steps for MeUmb $(Manp)_2$ with those for MeUmb-Man*p*, since both kinetic steps of the alternating model are bimolecular. Concerning the two possible positions of the MeUmb group, the one at X for MeUmb $(Manp)_2$, bound as C_a , corresponds best to the MeUmb-Man*p* complex, whereas K_a is about equal to the binding constant for Me-Man*p*. This suggests that the interactions of the MeUmb group with the protein are quite different for MeUmb-Man*p* and MeUmb $(Manp)_2$ and/or that for the latter, the presence of a terminal Man*p* disturbs the specific recognition of the Man*p* group adjacent to the MeUmb group. The two suggested binding mechanisms are interconvertible, and are two extremes of a more general mechan-

ism, which is obtained by inserting an equilibrium converting C_a and C_b in the alternating scheme in table 2. All the results of temperature-jump experiments are consistent with those of the stopped-flow study in table 2.

Stopped-flow traces for the binding of MeUmb (Man*p*)$_3$ to Con A (up to 94 μM) were always triphasic with time constants in the ranges 0.14–0.27 sec, 2.3–0.8 sec and 10–17sec. The fastest process showed the smallest amplitude. The slower phases were difficult to observe by the time available for our temperature-jump technique (<1 sec).

These results are consistent with the recent stopped-flow data which show biphasic progress curves for other"bivalent" carbohydrates such as *p*-NO_2 Phe Man*p* α (1 → 2) Man*p* and*p*-NO_2 Phe Glc*p* α (1 → 2) Man*p* but that are monophasic for *p*-NO_2 Phe Gal*p* α (1 → 2) Man*p* (Williams *et al.,* 1978b, 1981). The latter is not unexpected since simple galactosides do not bind to Con A.

Factors that can increase the affinity of glycosides: From the above kinetic results and in view of the practically constant values of the binding enthalpies in table 1, it appears that all carbohydrates Me-Man*p* (Man*p*)$_n$ and MeUmb (Man*p*)$_n$ bind to Con A with only one Man*p* residue. Yet, the association constants increase markedly within the series (Man*p*)$_n$ as *n* increases (K_n /K_1 = 1, 1.9 and 17 for Me-Man*p* and *n* = 2 and 3 in table 1). A statistical reason for this increase in affinity, representing the increase in the possibilities for binding the available Man*p* residues, does not totally account for the largest value. In addition, the results of table 2 indicate a large binding preference for the terminal Man*p* in MeUmb (Man*p*)$_2$. Unspecific interactions could account for these discrepancies and some of these could originate from the aglycon. In addition to the increase in *K* with the MeUmb group (*e.g.* with MeUmb-Man*p* as compared to Me-Man*p*), some *p*-substituted Phe α-Man*p*, with electron releasing substituents bind 4 to 5 times better (Loontiens *et al.,* 1973, 1975) than (Man*p*)$_2$. Such unspecific interactions could also apply to a nonbinding carbohydrate like Gal*p* since *p*-NO_2 Phe Gal*p* α (1 → 2) Man*p* (Williams *et al*.,1981) is a better ligand than *p*-NO_2 Phe α-Man*p* or *p*-NO_2 Phe α-2-OMeMan*p* (Williams *et al.*, 1978b, 1981). Unspecific interactions can also occur in *p*-substituted Phe β-glucosides; the corresponding linear free energy relationships with the molecular refractivity of the substituent (Hansch, 1975; Hansch and Calef, 1976) are superior to those with the hydrophobicity parameter (Loontiens *et al.*, 1973). This can arise from interaction with amphiphilic side chains at the surface of a protein.

Conclusions from kinetic studies with Con A: It is interesting to compare the binding kinetics of all the carbohydrates investigated so far; this is done in table 3 together with the recent data of Farina and Wilkins (1980) and Williams *et al.* (1981) The carbohydrates involved are the α and β Me-glycosides of glucose and methyl α-mannoside, arylglycosides and aryldisaccharides. For all these ligands an apparent single-step association is valid; this also holds for a disaccharide if the individual formation of C_a or C_b in table 2 is considered. The association-rate parameters are comparable and low (3×10^4 to 11×10^4 M^{-1} sec^{-1}). This is about four orders of magnitude smaller than can be predicted for a diffusion-controlled process and the high activation energy (63 $kJmol^{-1}$) for binding of MeUmb-Man*p* is also inconsistent with a diffusion-controlled reaction (Clegg *et al.,* 1977).

Table 3. Comparison of the reaction-rate parameters (k_+ and k_-) and kinetically defined association constants (K) for the binding of carbohydrates to Con A according to a bimolecular one-step association

	$10^{-4} \times k_{+1}$ (M^{-1} sec^{-1})	k_{-1} (sec^{-1})	$10^{-3} \times K$ (M^{-1})	t (°C)	pH
MeUmb (Man*p*)$_2$[a]	2.8	0.12	240	20	5.5
	3.6	10.1	3.5	20	5.5
p-NO_2 (Man*p*)$_2$[b]	3.2	0.22	145	25	5.0
	2.3	1.1	21	25	5.0
MeUmb-Man*p*[c]	10.2	3.2	32	24.1	7.2
	11.3	3.4	33	24.1	5.5
p-NO_2 Phe-Man*p*[d]	5.4	6.2	8.7	25	5.0
p-NO_2 Phe-2-O-MeMan*p*[b]	6.6	8.2	8.1	25	5.0
Me-Man*p*[e]	4	12	3.3	23.2	7.2
Me-Glc*p*[e,f]	5	31	1.7	25	7.2; 5.6
MeβGlc*p*[f]	2.8	400	0.07	25	5.6

[a] Van Landschoot *et al*., 1980b; [b]Williams *et al.*, 1978b; [c] Clegg *et al*.,1977; Loontiens *et al.,* 1977b; [d]Lewis *et al.,* 1976; [e]Clegg *et al.*, 1981; [f]*Brewer et al.*, 1973, 1974

Association-rate constants for carbohydrate binding to proteins are usually low (Pecht and Lancet, 1977; Clegg *et al.,* 1981) which could be related to the hydrophylic nature of these ligands. For Con A, the comparably slow rate for all ligands points at a common rate -determining step. Theoretically this could arise from a rapid isomerisation in either of the free reaction partners themselves, provided that the concentration of the reacting species were very low; this case is improbable for Con A in view of the controls that were made (Clegg *et al*.,1977). An alternative possibility is a rapid formation of the Con A-carbohydrate complex that is possibly diffusion controlled and consequently undergoes a slow conformational change in the observed time window. This two-step mechanism, for which we could not find any evidence by absorption, fluorescence and polarisation of fluorescence in the μ sec time range, could involve a local change in tertiary protein structure, that comprises hydrogen bond interactions within the protein and with water molecules. Such a conformational change has been postulated from CD (Pflumm *et al*.,1971; Cardin *et al*.,1979) NMR stopped-flow (Grimaldi and Sykes, 1975) and also from infusion of Me-Man*p* in Con A crystals (Hardman and Ainsworth, 1973).

A possible biological consequence can be formulated from the rate parameters in table 3. Since the association-rate parameters are comparable, it is the dissociation-rate parameter that determines the association constant: for a poor ligand like Me-β-Glc*p* dissociation is much faster (400 sec^{-1}) than for a good ligand like MeUmb (Man*p*)$_2$ (with *e.g.* 0.1 sec^{-1}). The slow complex formation, that is characteristic for Con A, as well as the slow dissociation rate of good ligand result in an appreciable lifetime of a complex without imposing the restrictions of a very high affinity. Such a long residence time of an initially formed Con A – receptor com plex could be related to the effectiveness for a topological reorganisation when

several glycoconjugate receptors interact with a multivalent Con A molecule. A prerequisite for these events to occur would be a sufficiently long lifetime of the initial complex to allow other Con A receptors to form additional interactions by the slow process of lateral diffusion in the membrane.

WGA⋆

Equilibrium studies with WGA: The binding of carbohydrates to WGA is more complex than to Con A. It has been shown by X-ray diffraction (Wright, 1980a, b) that in the dimeric WGA with M_r = 18000 each protomer possesses a primary and a secondary binding site. Sialic acid binds to the primary site only, whereas (GlcNAc*p*)$_2$ binds to both sites. Carbohydrates larger than (GlcNAc*p*)$_2$ could not bind to either site in the crystals due to space limitations.

In solution the binding of MeUmb-GlcNAc*p* and WGA, determined by equili brium dialysis and titrations using difference absorption spectra (Van Landschoot *et al.,* 1977), is simple throughout the saturation range and corresponds to four identical and independent binding sites in the dimeric lectin. These results are consistent with those for reduced (GlcNAc*p*)$_4$ (Privat *et al.,* 1974a). Binding studies with MeUmb (GlcNAc*p*)$_2$ and MeUmb (GlcNAc*p*)$_3$ are only possible at low fractional saturation (r < 0.4), probably due to association of lectin molecules yielding insoluble complexes that can be redissolved with an excess of (GlcNAc*p*)$_3$. The properties derived from the binding experiments with MeUmb (GlcNAc*p*)$_n$ and WGA under these restrictive conditions are given in table 4, Unlike Con A, the value of $-\Delta H°$ for binding of WGA to MeUmb (GlcNAc*p*)$_2$ is noticeably larger than for binding of the monosaccharide. The fluorescence of both ligands is totally quenched when they bind to WGA and this, together with the partial quenching for MeUmb (GlcNAc*p*)$_3$ fluorescence, suggests a preliminary binding scheme (III) given with table 4. However, the different values of $\Delta\varepsilon$ for the similar difference absorption spectra obtained with MeUmb-GlcNAc*p* and MeUmb (GlcNAc*p*)$_2$, given in table 4, suggest that this scheme is too simple (see table 5).

The relative values of the association constants for MeUmb (GlcNAc*p*)$_n$ are difficult to compare with those of (GlcNAc*p*)$_n$ since the increase in affinity with n for the latter ligands varies from 1, 6.5 and 29 (Privat *et al*.,1974c) to 1,800 and 3000 (Allen *et al*., 1973) among the reported values obtained by different methods (Lotan and Sharon, 1973; Nagata and Burger, 1974; Goldstein *et al*.,1975; Bhavanandan *et al.,* 1979; Peters *et al*., 1979). This could be due to experimental problems caused by a complex binding phenomenon which is suggested by the concave Scatchard plot for the binding of (GlcNAc*p*)$_3$ and WGA isolectin I (Allen *et al.* ,1973), in figure 2. Such conclave plots were also obtained with MeβGlcNAc*p* and (GlcNAc*p*)$_2$ and were independent of whether individual isolectins or a mixture of isolectins were used. When 50% displacement of MeUmb (GlcNac*p*)$_2$ was taken as an indication for the affinities of Me-βGlcNAc*p*, (GlcNAc*p*)$_2$ and (GlcNAc*p*)$_3$, these relative values were 1, 15 to 20 and 110 to 120 at 14 to 18° C (Van

⋆ All equilibrium and kinetic data for binding of carbohydrates to WGA were obtained using 0.1 M NaAc/Hac, 0.5 M NaCl at pH 4.7 between 10 and 40° C. WGA concentrations were expresed as sites on the basis of an equivalent M_r equal to 9000.

Table 4. Characteristics for the binding of MeUmb $(GlcNAcp)_n$ to WGA at 25° C.

	$10^3 \times \Delta\varepsilon_{318}$ $(M^{-1}\ cm^{-1})$	F_∞/F_0	$10^{-4} \times K$ (M^{-1})	$-\Delta H°$ $(kJmol^{-1})$	$-\Delta S°$ $(Jmol^{-1}\ K^{-1})$
MeUmb-GlcNAc*p*	−2.77	−0.01 ±0.02	2.0 ± 0.1	34.3 ± 1.7	32.7 ± 6.3
MeUmb $(GlcNAcp)_2$	−1.07	−0.01 ±0.02	7.5 ± 0.3	41.9 ± 1.3	46.9 ± 4.2
MeUmb $(GlcNAcp)_3$	0	0.56 ±0.02	11.7 ± 0.6	35.2 ± 1.3	20.1 ± 5.0

Δε refers to the change in absorption at 10.8° C for difference absorption spectra that were similar in form for MeUmb-GlcNAc*p* and MeUmb $(GlcNAcp)_2$. The values of F_∞/F_0 and the binding parameters were obtained from fluorescence titrations with an excess of WGA at nine to eleven temperatures. For MeUmb-GlcNAc*p* these results were consistent with those obtained by equilibrium dialysis and by titrations using the ligand difference absorption spectra (Van Landschoot *et al.*, 1977).

Preliminary binding scheme:

A B C D
G – F
G – G – F
G – G – G – F

(Scheme III)

with G = GlcNAc and F = MeUmb.

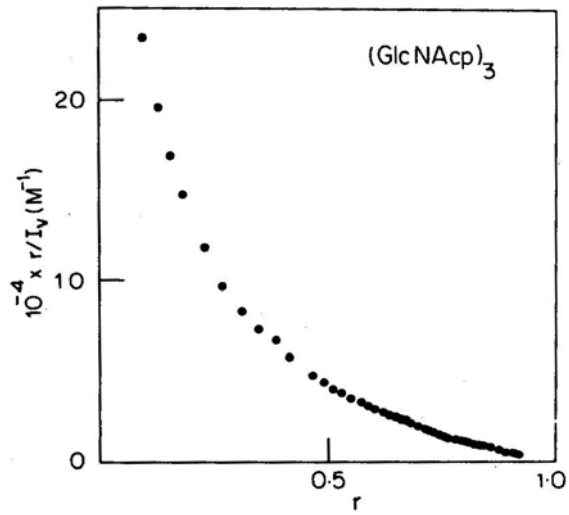

Figure 2. Scatchard plot for binding of $(GlcNacp)_3$ to WGA at pH 4.7 and 16°C.The data were obtained by a continuous substitution titration according to Van Landschoot *etal.* (1980a) using MeUmb $(GlcNAcp)_2$ as a fluorescent indicator ligand. To 2.948 ml of a mixture of 3.24 μM MeUmb $(GlcNAcp)_2$ and 29.1 μM WGA (isolectin I) in a 1 × 1 × 4.5-cm cuvette, 3.13 mM $(GlcNacp)_3$ was added from a microburette under continuous stirring. The fluorescence of MeUmb $(GlcNAcp)_2$, released from its totally quenched WGA complex was recorded. After correction for dilution, and for a blank without MeUmb $(GlcNAcp)_2$, the data were transformed into a Scatchard plot. An attempted fit for two different and independent binding sites was unsatisfactory (Van Landschoot, 1978).

Table 5. Reaction-rate parameters and characteristics for binding of MeUmb-GlcNAc*p* and MeUmb $(GlcNAcp)_2$ to WGA at 23.6° C and pH 4.7 according to two consistent mechanisms.

Sliding (sequential)	Alternating (excluded site)
$A+B \underset{-1\ \text{fast}}{\overset{+1}{\rightleftharpoons}} C_1 \underset{-2\ \text{slow}}{\overset{+2}{\rightleftharpoons}} C_2$	$C_a \underset{-a\ \text{fast}}{\overset{+a}{\rightleftharpoons}} A+B \underset{-b\ \text{slow}}{\overset{+b}{\rightleftharpoons}} C_b$
MeUmb-GlcNAc*p*: two relaxations (coupled)	
$k_{+1} = 3.0\times10^6\ M^{-1}\ sec^{-1}$	$k_{+a} = 1.5\times10^6\ M^{-1}\ sec^{-1}$
$k_{-1} = 1.8\times10^2\ secsec^{-1}$	$k_{-a} = 3.2\times10^2\ sec^{-1}$
$k_{+2} = 1.1\times10^2\ sec^{-1}$	$k_{+b} = 1.5\times10^6\ M^{-1}\ sec^{-1}$
$k_{-2} = 70\ sec^{-1}$	$k_{-b} = 39\ sec^{-1}$
$K_1 = k_{+1}/k_{-1} = 1.7\times10^4\ M^{-1}$	$K_a = k_{+a}/k_{-a} = 4.7\times10^3\ M^{-1}$
$K_2 = k_{+2}/k_{-2} = 1.6$	$K_b = k_{+b}/k_{-b} = 3.8\times10^4\ M^{-1}$
$K_I = K_1(1+K_2) = 4.3\times10^4 M^{-1}$	$K_{II} = K_a+K_b = 4.3\times10^4\ M^{-1}$
MeUmb $(GlcNAcp)_2$: two relaxations (uncoupled)	
$k_{+1} = 1.0\times10^7\ M^{-1}\ sec^{-1}$	$k_{+a} = 9.3\times10^6\ M^{-1}\ sec^{-1}$
$k_{-1} = 2.0\times10^2\ sec^{-1}$	$k_{-a} = 2.3\times10^2\ sec^{-1}$
$k_{+2} = 21\ sec^{-1}$	$k_{+b} = 1.1\times10^6\ M^{-1}\ sec^{-1}$
$k_{-2} = 18\ sec^{-1}$	$k_{-b} = 16\ sec^{-1}$
$K_1 = 5\times10^4\ M^{-1}$	$K_a = 4.0\times10^4\ M^{-1}$
$K_2 = 1.17$	$K_b = 6.9\times10^4\ M^{-1}$
$K_I = K_1(1+K_2) = 1.1\times10^5 M^{-1}$	$K_{II} = K_a+K_b = 1.1\times10^5\ M^{-1}$
$\Delta\varphi_2 = 0$ (assumptions)	$\Delta\varphi_a = \Delta\varphi_b$
$\Delta H_1 = -33\ kJmol^{-1}$	$\Delta H_a = -36.8\ kJmol^{-1}$
$\Delta H_2 = -8.1\ kJmol^{-1}$	$\Delta H_b = -37.5\ kJmol^{-1}$
$\Delta H_I = \Delta H_1 + \frac{K_2}{1+K_2}\Delta H_2 = -37.3\ kJmol^{-1}$	$\Delta H_{II} = (K_a\Delta H_a + K_b\Delta H_b)\times(K_a+K_b)^{-1} = -37.3\ kJmol^{-1}$

The data were obtained by the temperature-jump relaxation technique at 20.4 + 3.2° C measuring the ligand fluorescence. The kinetic data were obtained under the following conditions: 2.1 μM MeUmb-GlcNAc with 5 to 240 μM WGA or 2.6 μM MeUmb $(GlcNAcp)_2$ with 2 to 80 μM WGA. The relaxation curves were always bi-exponential. For MeUmb-GlcNAc*p* the faster relaxation (with relaxation times of 3.0 to 1.6 m sec) showed a maximal amplitude at about 25 μM WGA and the slower relaxation (19 to 3 m sec) had a maximal amplitude at about 100 μM WGA. The results with MeUmb $(GlcNAcp)_2$ were similar: the faster relaxation (4 to 1 m sec) had its maximal amplitude at 6 μM WGA and the slower one (55 to 10 m sec) was maximal at about 14 μM WGA. The reaction-rate parameters were calculated from linear plots of the sum of the product of the inverse relaxation times *vs* the free concentration of ligand and protein. For MeUmb-GlcNAc*p*), the concentration dependence of the relaxation times was analyzed for kinetically coupled steps 1 and 2. For MeUmb $(GlcNAcp)_2$ an analysis for uncoupled steps was completely satisfactory and this property facilitated the calculation of ΔH values from the relative amplitudes (the kinetic change in fluorescence divided by the static fluorescence). It was assumed that ligand fluorescence φ was totally quenched in all complexes. ΔH_1 and ΔH_a were obtained from the slope of a linear plot for the relative amplitude of the faster process *versus* its relaxation time; ΔH_I and ΔHII, the kinetically defined overall binding enthalpy, was obtained from the slope of a linear plot for the total relative amplitude *versus* the product of the two relaxation times. For MeUmb $(GlcNacp)_2$ the kinetically defined association constant (1.1×10^5 M^{-1}) and binding enthalpy (–37.3 $kJmol^{-1}$) were in fair agreement with the values 8.0×10^4 M^{-1} (at 23.6°C) and – 41.9 $kJmol^{-1}$ obtained at equilibrium.

Landschoot, 1978). Deviations from a simple binding curve, similar to figure 2 have also been observed by Midoux (1980) for the binding of Me-β (GlcNAc*p*)$_2$ and WGA at pH 7.2; as measured by the enhancement of tryptophan fluorescence, the curvature of the Scatchard plots was very pronounced for 5 μM WGA, and gradually disappeared when WGA concentrations were increased to 160 μM.

The apparent affinity of Me-β (GlcNAc*p*)$_2$ decreases by a factor larger than 10 as the concentration of WGA increases. These complex binding phenomena have been related to aggregation of WGA (Midoux, 1980) and to a monomer-dimer equilibrium of WGA that depends on its concentration, pH and binding of carbohydrates (Midoux, 1980).

Kinetic studies with WGA: The progress curves of the kinetic experiments obtained with MeUmb (GlcNAc*p*)$_n$ and WG0A were faster than those for Con A, and could conveniently be studied by the temperature-jump relaxation technique. The results (Van Landschoot *et al.,* 1978b; Clegg *et al*.,1980, in preparation) are summarized in table 5. Unlike the previously discussed results obtained with Con A, both the mono- and disaccharides MeUmb-GlcNAc*p* or MeUmb (GlcNAc*p*)$_2$ show biphasic kinetic processes when binding to WGA. This, together with the values of Δ H° for the .binding of the mono- and disaccharides, (table 4) suggests that the binding region contains two subsites in WGA.

The biphasic behaviour of the relaxation times for the binding of WGA to both ligands MeUmb-GlcNAc*p* and MeUmb (GlcNAc*p*)$_2$ is similar; the inverse of the relaxation time of the faster process increases linearly with concentration, whereas the slower one increases hyperbolically, approaching a constant value at high concentrations. This behaviour is consistent with the two mathematically equivalent models in table 5. Here, as in the case of Con A, these two models can be regarded as a binding followed by a sliding over the site, or as two alternative bimolecular binding processes. For the latter mechanism, the deduced values of ΔH_a and ΔH_b for MeUmb (GlcNAc*p*)$_2$ are virtually the same. In the sliding mechanism however, fast binding in step 1 has a value of $-\Delta H_1$, that is much larger than $-\Delta H_2$ for the slow monomolecular process in step 2. Both complexes C_1 are almost equally populated at equilibrium. This value of $-\Delta H_2$ for the sliding mechanism nearly accounts for the difference in the equilibrium constants for MeUmb-GlcNAc*p* and MeUmb (GlcNAc*p*)$_2$ in table 4; this can be interpreted as the contribution of the second GlcNAc*p* residue to the equilibrium value of Δ H for MeUmb (GlcNAc*p*)$_2$. An attempted refinement of scheme (III) is represented in figure 3. This was done considering the signal changes in table 4 and the values of

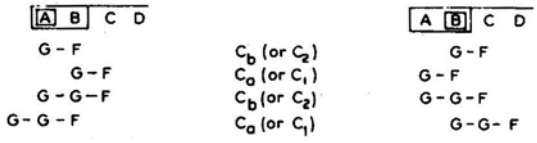

Figure 3. Suggested possible binding positions of MeUmb (GlcNAc*p*)$_n$ [G_n F] at a binding region of WGA composed, of two subsites A and B with different affinities for GlcNAc*p*. Left: the binding region is "open" at the left of A, the affinity at A is larger than at B, sites B and C cause 100% quenching of the MeUmb group F; at B, the value of – Δε is larger than at C. Right: the binding region at the left of A is not accessible to G, the affinity at B is larger than at A, sites B, C and D cause 100% quenching of group F and the value of – Δε decreases in the direction of B, C, D

the association constants per step in table 5. It should be noted that the two possibilities in figure 3 differ in the position of the subsite with the highest affinity. It is difficult as yet to compare our results for MeUmb (GlcNAc*p*)$_r$ with those from X-ray diffraction data for (GlcNAc*p*)$_n$ (Wright, 1980b) in view of the different binding properties of these two ligand series, both in the crystals and in solution.

The binding kinetics of MeUmb (GlcNAc*p*)$_3$ and WGA showed a total of four relaxations of fluorescence. The two faster ones had positive amplitudes similar to the two ligands discussed above and the amplitude of the fastest one became unobservable for concentrations to WGA larger than 10 μM. The values and the concentration dependencies of these two relaxation times were similar to those for MeUmb-GlcNAc*p* and MeUmb (GlcNAc*p*)$_2$. At concentrations higher than 10 μM however, two additional relaxations appeared. An analysis of the relaxation times shows that the simple alternative binding mechanism is not valid for MeUmb (GlcNAc*p*)$_3$. It should still be established if these complex kinetics of MeUmb (GlcNAc*p*)$_3$ are related to the impaired accessibility of the binding region f WGA for the higher members of the chito-oligosaccharides derivatives (Wright, 1980b), or to higher aggregations of the protein-oligosaccharide complexes.

Concluding remarks

The combination of equilibrium and kinetic measurements with Con A and WGA have resulted in highly informative studies of the carbohydrate-protein interactions. The intense signal changes observed with the fluorescent MeUmb-glycosides of the simple sugars and of the low molecular oligosaccharides are advantageous for these experiments, especially the kinetic studies. For all α (1 → 2)-linked manno-oligosacchrides, Con A binds a single Man*p* residue, whereas for chito-oligosaccharides, WGA binds two adjacent GlcNAc*p* residues. For all carbohydrates that bind to Con A, there seems to be a common, rate-determining association step, resulting in similar and characteristically low association rates, whereas the slow but variable dissociaton rates determine the association constants. These two slow rates are probably a favourable factor for the biological action of these lectins when one considers the slow rearrangements in the membrane leading to multiple cell-wall receptor-lectin complexes.

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Note added in proof

The quenching of MeUmb-Man*p* fluorescence upon binding of this ligand to Con A has been further exploited as an indicator system to study binding of the required metal ions [Koenig, S. H., Brown, R. D. III, Brewer, C. D. and Sherry, A. D. (1982) *Biochem. Biophys. Res. Commun.,* **109,** 1047–1053; Sophianopoulos, A. J. and Sophianopoulos, J. A. (1983) *Arch. Biochem. Biophys.,* **223,** 350] and to investigate carbohydrate binding to monomeric Con A [Sophianopoulos, A. J. and

Sophianopoulos, J. A. (1982) *Arch. Biochem. Biophys.,* **217,** 751–754]. We have recently published a more complete analysis of the kinetic data on WGA [Clegg, R. M, Loontiens, F. G., Sharon, N. and Jovin, T. M . (1983) *Biochemistry,* **22,** 4797–4804]. As to the versatility of 4-methylumbelliferyl glycosides in binding studies with lectins, MeUmb difference absorption spectrometry seems to be of general applicability since it enables monitoring of binding with all of the ten lectins investigated to date [De Boeck, H., Lis, H., Sharon, N. and Loontiens, F. G. (1983) *15th FEBS Meeting Brussels* (*Belgium*)*,* July 24–29, p. 213] . In this respect we have reported on a continuous titration technique of differences absorption [De Boeck, H., Loontiens, F. G. and De Bruyne, C. K. (1982) *Analyt: Biochem.,* **124,** 308–313] Detailed studies on the binding of MeUmb Galβ (1→3) GalNAc to the lectin from peanut have dealt with aspects of equilibrium [Decastel, M., Tran, A.-T. and Frénoy, J. P. (1982) *Biochem. Biophys. Res. Commun.,* **106,** 638–643; De Boeck, H., Matta, K. L., Claeyssens, M., Sharon, N. and Loontiens, F. G. (1983) *Eur J. Biochem.,* **131,** 453–460] and stopped-flow kinetics [Loontiens, F. G. (1983) *FEBS Lett,* **162,** 193–196] Similar approaches of lectin-carbohydrate interaction with the lectin from soybean [De Boeck, H., Loontiens, F. G., Lis, H. and Sharon, N. (1983) *15th FEBS Meeting Brussels* (*Belgium*), July 24–29, p. 213] will be published in detail or are reaching a final stage with the lectin from *Erythrina cristagalli.* With cellulolytic enzymes, 4-methylumbelliferyl cello-oligosaccharides are useful to investigate the degradative mechanisms [Van Tilbeurgh, H., Claeyssens, M. and De Bruyne, C. K, *FEBS Lett,* **149,** 152–156].

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