Mechanism of Binding of Mono- and Oligosaccharides to Concanavalin A: A Solvent Proton Magnetic Relaxation Dispersion Study†

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ABSTRACT: In previous studies of the interaction of solvent water molecules with the Mn^{2+} ion in manganese–concanavalin A (Ca^{2+}–Mn^{2+}–Con A) by observation of the magnetic field dependence (dispersion) of the spin–lattice relaxation rate (T_1^{-1}) of the solvent water protons over a wide range of magnetic fields [Koenig, S. H., Brown, R. D., & Brewer, C. F. (1973) *Proc. Natl. Acad. Sci. U.S.A. 70*, 475], we have shown that T_1^{-1} is dominated by the residence time of an exchanging water ligand(s) on the Mn^{2+} ion. Additional measurements were made on Ca^{2+}–Mn^{2+}–Con A solutions in the presence of sufficient amounts of either methyl α- or β-D-glucopyranoside to saturate the carbohydrate binding sites of the protein, and it was observed that the relaxation rate across the dispersion spectrum was reduced by approximately 15%. In the present study, we have measured the effects of binding of a series of mono- and oligosaccharides to Ca^{2+}–Mn^{2+}–Con A on the solvent water proton relaxation rate over a range of magnetic fields from 5 Oe to 12 KOe. The observed change in relaxation rate was shown to be sensitive to the affinity constants of the saccharides tested in that the effect was proportional to the amount of saccharide bound to the protein. Quantitative analysis revealed that the observed decrease in solvent relaxation rate upon saccharide binding is due to an increase in the residence time of the exchanging water ligand(s) of the Mn^{2+} ion. This effect is consistent with a conformational change in the protein upon binding of saccharides. We find that binding of methyl α- and β-D-glucopyranoside, methyl α-D-mannopyranoside, and *o*-iodophenyl β-D-glucopyranoside in sufficient amounts to saturate the carbohydrate sites of the protein produces the same increase in the residence time of the exchanging water ligand(s). Galactose and *o*-iodophenyl β-D-galactopyranoside, which do not bind under the same conditions, show no effects. The same change in the dispersion profile as that caused by the above monosaccharides was observed with the following oligosaccharides when added in sufficient amounts to saturate the carbohydrate binding sites of the protein: D-maltose, D-maltotriose, D-maltotetraose, *O*-α-D-mannopyranosyl-(1→2)-D-mannose, *O*-α-D-mannopyranosyl-(1→2)-*O*-α-D-mannopyranosyl-(1→2)-D-mannose, *O*-α-D-mannopyranosyl-(1→2)-*O*-α-D-mannopyranosyl-(1→2)-*O*-α-D-mannopyranosyl-(1→2)-D-mannose, and melezitose. Goldstein and co-workers [Goldstein, I. J., Reichart, C. M., & Misaki, A. (1973) *Biochim. Biophys. Acta 317*, 500] have shown that the first three oligosaccharides have nearly the same affinity as monosaccharides, whereas the α(1→2)-linked mannans show increasing affinity constants with increasing chain length. Melezitose also shows enhanced binding by a factor of three relative to methyl α-D-glucopyranoside. The data suggest, therefore, that all of the above mono- and oligosaccharides that bind to Con A induce the same protein conformational transition, as monitored by the dispersion measurements. Although the data do not rule out the possibility of an extended binding site in Con A, the argument is advanced that the above results as well as other data in the literature on carbohydrate–Con A interactions can be explained by a single saccharide residue binding site. The greater affinity of melezitose and the α(1→2)-mannose oligosaccharides is suggested to be due to an increase in the probability of binding associated with the presence of more than one binding residue in the oligomer chain and not to an extended binding site. This novel mechanism for protein–saccharide interactions is discussed in terms of the molecular properties of so-called "Con A receptors" on the surface of cells.

Interest in the protein concanavalin A (Con A),[1] a lectin isolated from the jack bean (*Canavalia ensiformis*), derives from its unusual biological properties. In particular, its ability to bind to the surface of both normal and transformed cells has made it a powerful tool for exploring a wide variety of cell-surface related biological effects (cf. Lis & Sharon, 1973). The interaction of Con A with cell-surface membranes is related to the saccharide binding properties of the protein. The saccharide binding specificity of Con A has been shown by Goldstein et al. (1965) to be directed toward the monosaccharides glucose and mannose, which contain similar hydroxyl group configuration at the 3, 4, and 6 positions. The protein binds the α anomers of these glycosides more strongly than the β anomers.

Since cell-surface carbohydrate determinants occur as oligosaccharides in the form of glycoproteins and glycolipids, it is important to understand the interaction of Con A with these larger complex molecules. Goldstein (cf. Goldstein et al., 1973) has shown that there exist essentially two classes of oligosaccharides that bind to Con A. The first class of oligomers demonstrates no enhanced binding to the protein relative to monosaccharides; the second class shows enhanced binding. Included in the first class are α(1→3)-, α(1→4)-,

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[1] Abbreviations used: Con A, concanavalin A with unspecified metal content; Ca^{2+}–Mn^{2+}–Con A, concanavalin A containing manganese at the S_1 site and calcium at the S_2 site; Ca^{2+}–Zn^{2+}–Con A, concanavalin A containing zinc at the S_1 site and calcium at the S_2 site; α- and β-MDG, methyl α- and β-D-glucopyranoside, respectively; α-MDM, methyl α-D-mannopyranoside; β-IPG, *o*-iodophenyl β-D-glucopyranoside; β-IPGal, *o*-iodophenyl β-D-galactopyranoside; α(1→2)-mannobioside, *O*-α-D-mannopyranosyl-(1→2)-D-mannose; α(1→2)-mannotrioside, *O*-α-D-mannopyranosyl-(1→2)-*O*-α-D-mannopyranosyl-(1→2)-D-mannose; NMR, nuclear magnetic resonance; NMRD, nuclear magnetic relaxation dispersion.

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$\alpha(1\rightarrow6)$-linked oligosaccharides which contain a nonreducing terminal glucose or mannose residue (Goldstein et al., 1965); in the second class are $\alpha(1\rightarrow2)$-linked mannose oligomers (So & Goldstein, 1968; Goldstein et al., 1974). The $\alpha(1\rightarrow2)$-linked trisaccharide of mannose, for example, has a 20-fold greater affinity constant than α-methyl D-mannopyranoside (So & Goldstein, 1968). The enhanced binding of such oligosaccharides has prompted speculation (So & Goldstein, 1968) that the carbohydrate-combining site of Con A may bind more than one saccharide residue. Thus, Goldstein and others (cf. Young & Leon, 1974; Kornfeld & Ferris, 1975) have suggested that the specificity of Con A binding to oligo- and polysaccharides may involve extended interactions of the protein with several carbohydrate residues.

In light of the interest in isolating so-called "Con A receptors" from the surface of a variety of cells, it is of considerable importance to determine the mode of interaction of not only simple monosaccharides but also more complex oligosaccharides with Con A in order to elucidate the complete saccharide binding specificity of the protein. The goal of this paper is to explain the mode of interactions of mono- and oligosaccharides to Con A. We report evidence that the carbohydrate-combining site of Con A accommodates only one saccharide residue and that the enhanced binding of certain oligosaccharides can be explained by a statistical argument. Based on these data, a new receptor theory for Con A–membrane interactions is proposed.

Materials and Methods

Preparation of Con A Derivatives. Ca^{2+}–Zn^{2+}–Con A was obtained from Miles Yeda. Ca^{2+}–Mn^{2+}–Con A was prepared as previously described (Koenig et al., 1973). Atomic absorption analysis of these two Con A preparations showed essentially equal amounts of the transition metal ion and calcium ions. Sample solutions (0.6 mL) contained Con A at the appropriate concentration in pH 5.60, 0.1 N potassium acetate buffer, μ = 1.0 in potassium chloride. The final protein concentration was determined spectrophotometrically by using $A_{1cm}^{1\%}$ = 12.4 at 280 nm (Yariv et al., 1968; Brown et al., 1977).

Saccharides. The $\alpha(1\rightarrow2)$-mannose oligosaccharides were gifts from Dr. Irwin Goldstein. Synthesis of *o*-iodophenyl β-D-glucopyranoside (β-IPG) and *o*-iodophenyl β-D-galactopyranoside (β-IPGal) will be reported elsewhere. The rest of the saccharides used in this study were obtained from commercial sources. The purity of the saccharides was tested by thin-layer chromatography, paper chromatography, and melting points, where appropriate.

Relaxation Measurements. Measurements of the magnetic field[2] dependence of the solvent water proton relaxation rate (T_1^{-1}), i.e., nuclear magnetic relaxation dispersion (NMRD), were made by the field cycling method previously described (Koenig & Schillinger, 1969; Hallenga & Koenig, 1976).

Relaxation Theory. The theory of magnetic relaxation and the procedure used for obtaining the relevant parameters are found in Koenig et al. (1973).

Results

The NMRD of solvent water protons in solutions of Ca^{2+}–Mn^{2+}–Con A, Ca^{2+}–Zn^{2+}–Con A, and free Mn^{2+} is shown in Figure 1. T_{1para}^{-1}, the paramagnetic contribution of the bound Mn^{2+} ion of the protein to T_1^{-1}, obtained by correcting the observed NMRD of the Ca^{2+}–Mn^{2+}–Con A solution for the water background (T_{1w}^{-1}) and the diamagnetic contribution of the protein experimentally determined from the NMRD of Ca^{2+}–Zn^{2+}–Con A, is shown in Figure 2, upper curve. When sufficient α-MDG is added to saturate the saccharide binding sites of Ca^{2+}–Mn^{2+}–Con A, T_{1para}^{-1} is reduced by approximately 25% (Figure 2, lower curve). The lines are from fits to NMRD theory (Koenig et al., 1973). The NMRD of Ca^{2+}–Zn^{2+}–Con A is unaffected by the addition of the saccharide.

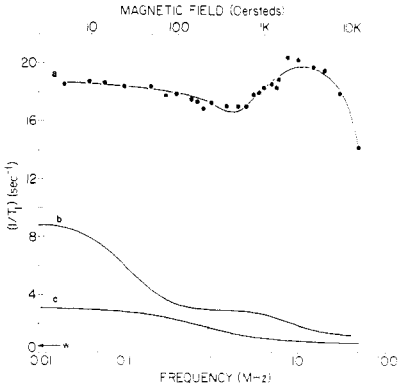

FIGURE 1: The magnetic field dependence of the spin-lattice relaxation rate of solvent water protons in solutions of (a) (top curve) Ca^{2+}–Mn^{2+}–Con A, 1.83 mM (monomer), 1.56 mM bound Mn^{2+}; (b) (middle curve) free Mn^{2+}, 0.15 mM; (c) (bottom curve) Ca^{2+}–Zn^{2+}–Con A, 1.83 mM. The field independent rate for pure water is indicated by *w*. All measurements were made at 25 °C in pH 5.60, 0.1 M potassium acetate buffer, μ = 1.0 in KCl. The solid curves are theoretical fits to data.

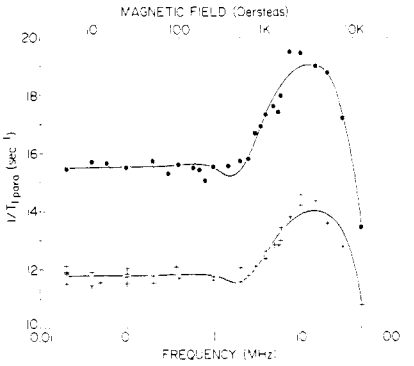

FIGURE 2: The paramagnetic contribution of the bound Mn^{2+} to the Ca^{2+}–Mn^{2+}–Con A dispersion shown in Figure 1 in (top curve) the absence of saccharide and (bottom curve) the presence of saturating (0.1 M) α-MDG. The solid lines are theoretical fits to the data.

When α-MDG is titrated into a solution of Ca^{2+}–Mn^{2+}–Con A, the change in T_{para}^{-1} at a given magnetic field reflects the fraction F_s of Con A with saccharide bound

$$F_s = \frac{T_{1para}^{-1}(S_0) - T_{1para}^{-1}(S_t)}{T_{1para}^{-1}(S_0) - T_{1para}^{-1}(S_s)} \quad (1)$$

where $T_{1para}^{-1}(S_0)$, $T_{1para}^{-1}(S_t)$, and $T_{1para}^{-1}(S_s)$ are the T_{1para}^{-1} values in the absence of α-MDG, in the presence of a given total concentration of the saccharide, and in the presence of a sufficient concentration of α-MDG to saturate the carbohydrate-binding site of the protein, respectively. Since the water and diamagnetic contributions are essentially independent of sugar concentration at the concentrations used, F_s can be determined from the observed T_1^{-1} values:

$$F_s = \frac{T_1^{-1}(S_0) - T_1^{-1}(S_t)}{T_1^{-1}(S_0) - T_1^{-1}(S_s)} \quad (2)$$

The slope of $1 - F_s$ plotted against $F_S(S_t - F_sP_t)$ (Figure 3), where P_t is the total Ca^{2+}–Mn^{2+}–Con A concentration, gives

[2] We indicate magnetic field intensity in units of the Larmor precession of frequency of protons in that magnetic field. The conversion is 4.26 kHz = 1 oersted = 1 gauss.

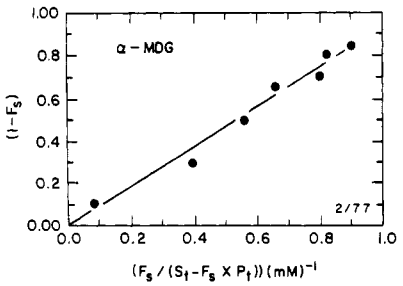

FIGURE 3: Plot for determining the association constant K_a for the interaction of α-MDG with Ca^{2+}–Mn^{2+}–Con A. F_s, the fraction of Ca^{2+}–Mn^{2+}–Con A molecules with saccharide, is determined from observation of the solvent proton relaxation rate at 0.04 MHz, as discussed in the text. P_t is the total Ca^{2+}–Mn^{2+}–Con A concentration, mM (monomer), and S_t the total saccharide concentration, mM. The slope of the line through the data gives a K_a of 1.1×10^3 M^{-1}. Measurements were made at 25 °C in pH 5.6, 0.1 M potassium acetate buffer, μ = 1.0 in KCl.

Table I: Saccharides Used to Determine the Effects of Mono- and Oligosaccharide Binding to Ca^{2+}–Mn^{2+}–Con A on the NMRD Profile of the Protein

methyl α-D-glucopyranoside
methyl β-D-glucopyranoside
methyl α-D-mannopyranoside
o-iodophenyl β-D-glucopyranoside
D-galactose
o-iodophenyl β-D-galactopyranoside
D-maltose
D-maltotriose
D-maltotetraose
O-α-D-mannopyranosyl-(1→2)-D-mannose
O-α-D-mannopyranosyl-(1→2)-*O*-α-D-mannopyranosyl-(1→2)-D-mannose
O-α-D-mannopyranosyl-(1→2)-*O*-α-D-mannopyranosyl-(1→2)-*O*-α-D-mannopyranosyl-(1→2)-D-mannose
melezitose

the association constant K_a (Steinhardt & Reynolds, 1969). The K_a value determined for α-MDG is 1.1×10^3 M^{-1}, in good agreement with values in the literature (McKenzie & Sawyer, 1973; Becker et al., 1975).

A variety of mono- and oligosaccharides (Table I) were tested for their effects on the NMRD of Ca^{2+}–Mn^{2+}–Con A. Figure 4 shows representative results for several of these saccharides when added in sufficient amounts to saturate the carbohydrate-binding sites of the protein. In each case, essentially the same decrease in the NMRD spectrum was observed. In fact, identical results were found for all of the saccharides listed in Table I with the exception of β-IPGal which has a low affinity constant and did not alter the NMRD at its maximum concentration, 20 mM. Titration of several of the mono- and oligosaccharides such as methyl β-D-glucopyranoside (β-MDG), melezitose, and maltotriose in solutions of Ca^{2+}–Mn^{2+}–Con A gave resulting K_a values that agree with previous estimates obtained by other techniques (cf. Loontiens et al., 1975). Titration of galactose into a solution of the protein at 25 °C yielded a K_a of 10 M^{-1}, consistent with its weak binding to Con A.

Quantitative determination of the effects of binding α-MDG, α(1→2)-mannobioside, and α(1→2)-mannotrioside on the NMRD of Ca^{2+}–Mn^{2+}–Con A is shown in Table II in terms of the parameters that determine T_{1para}^{-1}. The NMRD analysis followed the procedure of Koenig et al. (1973). The parameter which best describes the changes in the NMRD of the protein upon binding of these saccharides is τ_M, the residence time of the exchanging water molecules(s) on the manganese ion, which increases by ~40%. A smaller (~15%) change in τ_V is also observed. Changes in the other parameters

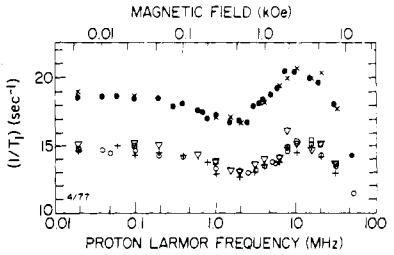

FIGURE 4: The magnetic field dependence of the spin–lattice relaxation rate of solvent protons in solutions of 1.56 mM Ca^{2+}–Mn^{2+}–Con A with no saccharide (●); with 25 mM of β-IPGal (×); and with saturating amounts of α-MDG, 6 mM (○); α-MDM, 10 mM (□); α(1→2)-mannobioside, 83 mM (▽), and α(1→2) mannotrioside, 15 mM (+). Measurements were made at 25 °C in pH 5.60, 0.1 M potasssium acetate buffer, μ = 1.0 in KCl.

Table II: Water-Ca^{2+}-Mn^{2+}-Con A Interaction Parameters[a] from Fit of Dispersion Theory to Data; Effect of Saturating Concentrations of Sugars ([Ca^{2+}-Mn^{2+}-Con A] = 1.56 mM; T 25 °C; pH 5.6)

sugar	r (Å) (±0.02)	$\tau_V \times 10^{-11}$ (s) (±0.4)	$\tau_{So} \times 10^{-10}$ (s) (±0.05)	$\tau_R \times 10^{-8}$ (s) (±0.6)	$\tau_M \times 10^{-6}$ (s) (±0.02)
none	2.30	9.08	1.51	4.88	1.43
α-MDG	2.29	7.69	1.33	6.34	1.96
α(1→2)-mannobioside	2.37	7.15	1.75		1.91
α(1→2)-mannotrioside	2.43	7.97	1.82		1.97

[a] r, Mn-proton distance for the exchanging water; τ_V, correlation time which describes the magnetic field dependence of τ_s; τ_{So}, spin-lattice relaxation time of the Mn^{2+} electronic moment at zero magnetic field; τ_R, rotational correlation time; τ_M, residence time of the water on the Mn^{2+} ion on the protein.

of the fit (r, τ_{So}, and τ_R) are not considered significant; variation in r and τ_{So}, are correlated since these parameters enter into the theory as τ_{So}/r^6. The reason for the anomalously small value for r (~ 2.35 Å) compared with that for the aquoion (2.8 Å) will be discussed by S. H. Koenig and R. D. Brown III (to be published).

Discussion

NMRD Measurements of Ca^{2+}–Mn^{2+}–Con A. In an earlier report, Koenig et al. (1973) observed that the NMRD of Ca^{2+}–Mn^{2+}–Con A was perturbed upon addition of amounts of α-MDG sufficient to saturate the carbohydrate-binding sites of the protein. This suggested that changes in NMRD could be used to monitor the binding of saccharides to Con A. Measurements of T_1^{-1} of solvent water protons have been widely used to obtain information on the binding of organic ligands to paramagnetic metalloproteins (cf. Mildvan & Cohn, 1970).

Figure 1 shows the observed NMRD of Ca^{2+}–Mn^{2+}–Con A, Ca^{2+}–Zn^{2+}–Con A, and free Mn^{2+} ions in solution. The NMRD for Ca^{2+}–Mn^{2+}–Con A is observed to be distinct from the other two curves. A quantitative analysis of the NMRD of Ca^{2+}–Mn^{2+}–Con A in terms of the parameters that enter into the theory of magnetic relaxation dispersion has been previously published (Koenig et al., 1973). Values of these parameters determined from a fit of this theory to the data are given in Table II. Of importance is that all of the manganese ions in the Ca^{2+}–Mn^{2+}–Con A solution are tightly bound to the protein, and that a water ligand(s) of the manganese ion in the protein is exchanging fairly rapidly (τ_M ~ 1.5×10^{-6} s) with bulk solvent to give the observed NMRD profile. Thus, there is a significant contribution of the residence time (τ_M) of the exchanging water molecule(s) to the

observed NMRD of solutions of Ca^{2+}–Mn^{2+}–Con A (Koenig et al., 1973). The Ca^{2+}–Zn^{2+}–Con A dispersion reflects a diamagnetic protein with the molecular weight of Con A in which the bulk solvent "experiences" the Brownian rotational motions of the protein (Hallenga & Koenig, 1976). The dispersion profile of free Mn^{2+} ions is shown to emphasize the different profile obtained for the ion when it becomes bound to Con A along with Ca^{2+} ions.

Binding of α-MDG to Con A. The magnetic field dependence of T_{1para}^{-1} in the absence of saccharide is shown in Figure 2, upper curve. When saturating amounts (0.1 M) of α-MDG are added to a solution of Ca^{2+}–Mn^{2+}–Con A, the T_{1para}^{-1} dispersion is reduced at all fields as shown in Figure 2, lower curve. In order to be sure that this change reflects binding of the monosaccharide to Con A and not nonspecific effects, the saccharide was titrated into a solution of Ca^{2+}–Mn^{2+}–Con A at 25 °C and the incremental changes in T_1^{-1} at 0.04 MHz were plotted as discussed above. The results are shown in Figure 3. A K_a value of 1.1×10^3 M^{-1} was obtained from the plot which agrees well with values obtained by equilibrium dialysis (McKenzie & Sawyer, 1973; Becker et al., 1975). We thus conclude that the change observed in the NMRD of Ca^{2+}–Mn^{2+}–Con A upon addition of α-MDG is a result of specific interactions between the protein and the saccharide.

A quantitative analysis of the change in the Ca^{2+}–Mn^{2+}–Con A dispersion upon addition of saturating amounts of α-MDG (Table II) indicates that the τ_M value of the exchanging water ligand of the manganese ion of the protein increases upon formation of the complex (i.e., the residence time of the exchanging water ligand on the ion becomes longer). Previous studies which used circular dichroism (Pflumm et al., 1971), kinetic rate measurements of ^{13}C enriched α-MDG binding to Ca^{2+}–Mn^{2+}–Con A (Brewer et al., 1974), and recent X-ray crystallographic data (Becker et al., 1976b) suggest a conformational change in the protein upon saccharide binding. We believe that the change in the NMRD spectrum of Ca^{2+}–Mn^{2+}–Con A upon binding α-MDG reflects this conformational change and that the increase in τ_m of the exchanging water ligand of the manganese ion reflects local changes in this region of the protein.

Koenig et al. (1973) have previously concluded that the change in the NMRD of Ca^{2+}–Mn^{2+}–Con A upon addition of α-MDG indicates that the saccharide does not bind directly to the manganese binding site in the protein. This conclusion was supported by the ^{13}C NMR data of Brewer et al. (1972, 1973a,b) which show that ^{13}C-enriched α-MDG binds 10–12 Å away from the manganese ion in the protein–saccharide complex. Subsequent NMR studies by Villafranca & Viola (1974) and Alter & Magnuson (1974) confirm these results. Although earlier X-ray crystallographic studies reported the carbohydrate-binding site to be located 20 Å from the manganese ion site in the protein (Edelman et al., (1972), recent X-ray diffraction results (Hardman & Ainsworth, 1976; Becker et al., 1976a) are now in agreement with the NMR findings. These latter crystallographic studies show the binding site to be a shallow depression on the surface of the protein approximately 5 Å from the calcium site and 10–13 Å from the manganese ion site. Furthermore, Becker et al. (1976b) have obtained additional results, indicating that some groups in the protein near the saccharide binding site move up to 6 Å upon saccharide binding to crystalline Con A. The above studies, therefore, support our conclusions that changes in the NMRD profile of Ca^{2+}–Mn^{2+}–Con A upon binding of α-MDG to the protein reflect a conformational transition in the protein and not direct binding of the saccharide to the manganese ion. These observations together with the recent reports by Brown et al. (1977) and Koenig et al. (1978) which demonstrate interactions of the metal ion binding sites (S1 and S2) and the saccharide-binding site of Con A indicate that the NMRD spectrum can be used as a sensitive monitor of the binding of saccharides to Ca^{2+}–Mn^{2+}–Con A.

Relative Binding Modes of Monosaacharides and Oligosaccharides to Con A. In order to examine the binding specificity of Con A, a comparative study of the binding of mono- and oligosaccharides to the protein was carried out by using the NMRD profile of Ca^{2+}–Mn^{2+}–Con as an index of the conformational change in the protein induced upon saccharide binding. The rationale for the experiments follows the observations made by Teichberg & Shinitsky (1973) who observed that oligosaccharides of increasing length and affinity that bind to lysozyme produce different conformational changes in the protein, as detected by fluorescence quenching of aromatic residues near the combining site. As more contacts were made by larger oligosaccharides in the combining site cleft of lysozyme, which is believed to accommodate up to six saccharide residues, the protein underwent concomitant steric adjustments. Similar observations have been made for wheat germ agglutinin which also binds oligosaccharides (Privat et al., 1974). By analogy, if oligosaccharides with enhanced binding activity toward Con A have additional binding contacts with the carbohydrate combining site, then additional conformational changes might occur in the protein.

The saccharides tested for their effect on the NMRD profile of Ca^{2+}–Mn^{2+}–Con A are listed in Table I. They include the β anomer of glucose, methyl β-D-glucopyranoside (β-MDG), which binds a factor of 25-fold less than the α anomer, and has been suggested by Brewer et al. (1973b) to have a different binding orientation to Con A than α-MDG. α-MDM was also included since it possesses the highest known affinity constant for Con A for a monosaccharide. Of the oligosaccharides tested, maltose, maltotriose, and maltotetraose represent a major class of oligomers which bind to the protein with affinity constants nearly the same as that of the corresponding monosaccharide, α-MDG. In addition, several α(1→2)-mannose oligomers were studied since these oligosaccharides show enhanced binding to the protein. Melezitose, *O*-α-D-glucopyranosyl-(1→3)-*O*-β-D-fructofuranosyl-(2→1)-*O*-α-D-glucopyranoside, has been demonstrated by Goldstein et al. (1965) to show enhanced binding by a factor of approximately 3 relative to α-MDG. The arylglucoside, β-IPG, has been shown by Brewer et al. (1973b) to bind to the saccharide-binding site of Con A in solution, but the corresponding aryl galactoside, β-IPGal, does not. Both of these arylglycosides bind to the "aromatic" binding site in the protein in the crystalline state (Hardman & Ainsworth, 1973). D-Galactose, which binds weakly to Con A, was also tested as a control.

Representative results of the effect of binding saturating amounts of several of the saccharides listed in Table I on the NMRD profile of Ca^{2+}–Mn^{2+}–Con A are shown in Figure 4. As can be observed, the results were essentially the same for α-MDG, α-MDM, α(1→2)-mannobioside, and α(1→2)-mannotrioside. The results, in fact, were the same for all of the saccharides tested in Table I with the exception of β-IPGal which binds only very weakly to the protein.

Table II indicates that τ_M is the parameter which changes significantly and that this change is the same when α-MDG, α(1→2)-mannobioside, or α(1→2)-mannotrioside binds to Ca^{2+}–Mn^{2+}–Con A. The data indicate, therefore, that the saccharides listed in Table I which bind to Ca^{2+}–Mn^{2+}–Con

A produce the same change in the residence time of the exchanging water ligand(s) of the Mn^{2+} ion of the protein. Since this change in τ_M appears to be associated with a conformational transition in the protein, the argument can be advanced that all of the saccharides that bind to Con A in Table I produce essentially the same conformational changes in the protein upon binding.

An alternative possibility is that our NMRD measurements of Ca^{2+}–Mn^{2+}–Con A are not sensitive to additional binding interactions that might take place between oligosaccharides such as α(1→2)-mannotrioside, which binds a factor of 20 times better than α-MDM, and the protein. Thus, the NMRD data do not rule out the possibility of an extended binding site in Con A. The very small changes that take place in the circular dichroism spectrum of the protein upon saccharide binding (Pflumm et al., 1971) do not appear to be a promising way to answer this question. However, another criterion can be used concerning extended interactions that may be occurring between the protein and certain oligosaccharides. In the case of lysozyme and other proteins with extended combining sites, inhibitors or substrates that bind to these proteins all possess very similar steric features regarding their overall conformations. This is demanded by the steric constraints of the combining sites of these proteins. Therefore, if Con A possesses an extended combining site, there should be equal constraints on the three-dimensional structures of saccharides that bind to the protein. However, space-filling models (CPK) of several of the oligosaccharides that show enhanced binding, such as α(1→2)-mannotrioside, melezitose, and the G1 glycopeptide isolated by Kornfeld & Ferris (1975), which shows enhanced binding, indicate little or no similarity in their overall conformations. It does not seen likely, therefore, that these oligosaccharides possess higher K_a values because of similar extended contacts with the protein. In fact, the small increments of enhanced binding of factors of 5 and 20 for α(1→2)-mannobioside and α(1→2)-mannotrioside, respectively, relative to α-MDM, do not compare with the large enhancements of 10^2 to 10^3 in binding that are observed for di- and trisaccharides, respectively, relative to monosaccharides, that bind to lysozyme (Banerjee & Rupley, 1973) which contains an extending binding site. These arguments also weigh against extended interactions between the binding site of Con A and oligosaccharides.

Our proposed interpretation of the data is that the mono- and oligosaccharides listed in Table I that bind to Con A all interact with the protein in the same manner. That is, since oligosaccharides produce the same change in the NMRD profile as monosaccharides, their binding modes must be quite similar. This would suggest that the oligosaccharides are binding through only one of their residues at any one time, in a manner similar to monosaccharide interactions with the protein. The enhanced binding of certain oligosaccharides can therefore be explained on a statistical basis. Of the oligosaccharides that possess enhanced binding constants, the α(1→2)-linked mannose oligomers are most evident. These oligomers differ from the maltose and isomaltose oligomers, which show no enhanced binding, in the position of their glycosidic linkages. As previously noted, Con A demonstrates binding specificity toward saccharides with glucose or mannose residues that posses free 3-, 4-, and 6-hydroxyl groups. Since oligosaccharides with α(1→3)-, α(1→4)-, or α(1→6)-glycosidic linkages possess modified hydroxyl groups at these critical binding positions, only the nonreducing terminal saccharide of these oligomers can bind to the protein, a conclusion reached by Goldstein and co-workers (1965). However, where the glycosidic linkage is α(1→2) for mannose oligomers, the internal residues also possess free 3-, 4-, and 6-hydroxyl groups. Goldstein et al. (1973) have shown that, when α(1→2)-mannotrioside was selectively modified at the first and third residues such that they could not bind to Con A, the resulting derivative containing an unmodified internal mannopyranoside residue was observed to bind as well as α-MDM. By using similar derivatization techniques, the reducing terminal residue of α(1→2)-mannotrioside was also shown to bind as well as free mannose. Goldstein thus concluded that internal α(1→2)-linked mannose residues could bind to Con A as well as nonreducing terminal residues. (It is interesting to note that, although each of the mannosyl residues of α(1→2)-mannotriose is individually capable of binding as well as the corresponding monosaccharide, the observed increment of enhanced binding to Con A is a factor of 20 instead of the many thousands of fold enhancement predicted from the combined binding potential of all three residues.)

Another oligosaccharide with enhanced binding is melezitose, which is structurally dissimilar to the α(1→2)-mannans. However, the two are similar in that melezitose also possesses more than one residue with free 3-, 4-, and 6-hydroxyl groups: the first and third glucose units. The enhanced binding of melezitose by a factor of seven relative to maltotriose, which contains only one "binding" residue at the nonreducing terminal end, is similar to the enhancement observed for α-(1→2)-mannobioside (which also contains two "binding" residues per molecule) relative to α-MDM. Thus, it appears that a necessary requirement for enhanced binding is for a molecule to contain multiple glucose or mannose residues which possess free 3-, 4-, and 6-hydroxyl groups.

We therefore suggest that the enhanced binding of certain oligosaccharides to Con A appears to be due not to an extended binding site on the protein, but rather to effects which result from clustering several "binding" residues (glucose or mannose) in the same molecule, any one of which can bind to the single residue site on the protein. To our knowledge, the effects of binding polyvalent ligands to monovalent protein binding sites has never been studied. Nevertheless, we feel the enhanced binding could come about from an increase in the forward rate constant for complex formation which, to the first approximation, would be expected to be proportional to the number of binding residues in the molecule. However, the observed enhancements for α(1→2)-mannobioside and α-(1→2)-mannotrioside of 4- and 20-fold, respectively, indicate more than proportional increases in their binding constants in relation to their number of "binding" residues. This suggests the possibility of decreased off-rates for these molecules as well. A possible mechanism for reducing the dissociation rates of these oligosaccharides may be one in which such a molecule upon dissociating from the protein is immediately recaptured by another of its binding residues. In effect, this would decrease the macroscopic off-rate of the oligosaccharide by limiting its diffusion away from the protein once binding has taken place. Such a recapture mechanism has been observed for the action of α- and β-amylases on polysaccharide substrates in which so-called multiple attacks occur on a single substrate oligomer (Abdullah et al., 1966; Thoma et al., 1971). We therefore suggest that a combination of these effects on the forward and reverse rates of binding of these oligosaccharides could account for their enhanced binding constants.

The absolute magnitude of the enhancement effects apparently reaches a limit between the tri- and tetrasaccharides in the α(1→2)-mannan series since higher homologues begin

to show decreased apparent binding constants toward Con A (Goldstein, 1975). However, this latter decrease may be due to the formation of intermolecular complexes of the higher oligomers in solution and not an actual decrease in their binding to Con A.

Evidence for a Single Residue Binding Site. A review of data in the literature on Con A–saccharide interactions is consistent with the protein possessing a single saccharide residue binding site. Goldstein and co-workers (Bessler et al., 1974) have demonstrated that a wide variety of monosaccharides, including α- and β-MDG, and oligosaccharides, including those with "internal" binding residues such as *O*-α-D-galactopyranosyl-(1→2)-*O*-α-D-mannopyranosyl-(1→2)-D-mannose, competitively displaces the chromogenic ligand *p*-nitrophenyl β-D-mannopyranoside from Con A. These workers concluded that the mono- and oligosaccharides used in the study competed for the same site on the protein. In addition, the specificity of the same site on Con A would require that all residues in a saccharide that bind directly to the protein possess the same molecular specificity as monosaccharides that bind to the protein. It is well known that the monosaccharides glucose and mannose bind well, as opposed to galactose which binds poorly, and that the α anomers of these monosaccharides bind better than the β anomers. Indeed, Goldstein et al. (1973) have shown that, when the terminal nonreducing residue of α(1→2)-mannotrioside is converted to a galactose residue, a loss in binding is observed. This result is consistent with the terminal nonreducing mannose residue α(1→2)-mannotrioside binding at the same site as α-MDM. Furthermore, Goldstein and co-workers (1965) have shown that reduction of mannose to give mannitol eliminates binding. When the terminal reducing end of α(1→2)-mannotrioside is correspondingly reduced to give α(1→2) mannotriotol, a loss in binding occurs which is also consistent with the terminal reducing residue of α(1→2)-mannotrioside binding to the same site as α-MDM. Similar substitutions affect the binding of another α(1→2)-linked oligosaccharide, kojibiose (*O*-α-D-glucopyranosyl-(1→2)-D-glucose). Kojibiose shows enhanced binding by a factor of three relative to *O*-α-D-galactopyranosyl-(1→2)-D-glucose (Goldstein et al., 1974). This suggests that the nonreducing residue of kojibiose contributes to binding, and the specificity of binding is similar to that observed for monosaccharide binding to Con A. Interestingly, the α-methyl anomer of kojibiose shows a further enhancement of binding by a factor of two relative to kojibiose, which is presumed to be a mixture of the α and β anomers. This suggests that the reducing end residue of the disaccharide binds to the protein at a site which also preferentially binds the α anomers of monosaccharides. The above data are all consistent with the protein containing a single binding site which can interact with either terminal nonreducing residues, internal residues, or terminal reducing residues of oligosaccharides providing that these glucose or mannose residues possess free 3-, 4-, and 6-hydroxyl groups.

A recent report by Van Landschoot et al. (1978) also provides evidence for the existence of a single carbohydrate-binding site on Con A. These workers have examined the binding of the 4-methylumbelliferyl derivatives of α-D-mannopyranoside, α(1→2)-mannobioside, and α(1→2)-mannotrioside to Con A by using difference absorption spectrometry and titration of ligand fluorescence quenching. Although the 4-methylumbelliferyl derivatized mannans do not show the same enhanced binding with increasing chain length as do the naturally occurring α(1→2)-mannans, the thermodynamic and spectral data for these fluorescent compounds are consistent with binding of a single α-D-mannopyranosyl residue of the oligomers to a single site on the protein. In addition, this group (Van Landschoot et al., 1977) has also reported preliminary results on the kinetics of binding of this series of 4-methylumbelliferyl α(1→2)-mannans to Con A. Their results show that the number of observed kinetic effects increases with the number of α-mannopyranosyl residues in the compound which, as suggested by the authors, could arise from an increase in the number of binding possibilities upon specific attachment that can occur through single mannopyranosyl residues of these compounds. These latter results agree with the biphasic association kinetics observed by Williams et al. (1978) for the interaction of *p*-nitrophenyl α(1→2)-mannobioside with Con A, by using stop-flow spectroscopic techniques. As contrasted with the monophasic association kinetics of *p*-nitrophenyl 2-*O*-methyl-α-D-mannopyranoside, the biphasic time course of binding of the corresponding α(1→2)-mannobioside was explained in terms of a model in which the disaccharide can bind to Con A in two different ways. Although the authors conclude that both mannopyranosyl residues of *p*-nitrophenyl α(1→2)-mannobioside interact simultaneously with the binding site of Con A, their data also are consistent with a statistical model for the association of the disaccharide with the protein in which either of the two mannopyranosyl residues can bind to the same single site on the lectin. The fact that biphasic association kinetics are observed for the disaccharide also provides evidence for the "recapture mechanism" that we have postulated for the dissociation behavior of the α(1→2)-mannans (above). It follows from the principle of microscopic reversibility that, if there is a rapid initial phase in the biphasic association reaction that represents the kinetically determined formation of two different complexes between Con A and *p*-nitrophenyl α-(1→2)-mannobioside, followed by a slow second phase in which the complexes equilibrate according to their equilibrium values, as suggested by the authors, then the off-rate for the disaccharide must follow the same kinetic pathway. That is, a given disaccharide molecule can reequilibrate between its two mannopyranosyl residues and the same binding site before the ligand completely dissociates from the protein. This may provide a mechanism for slowing the off-rates of these molecules which, in turn, helps explain their enhanced binding to Con A without invoking an extended binding site for the protein.

Thus, from consideration of all of the available evidence, it appears that the saccharide binding specificity of Con A can be accounted for by a binding site which accommodates only one saccharide residue, and that oligosaccharides containing multiple glucose or mannose residues which have free 3-, 4-, and 6-hydroxyl groups can demonstrate enhanced binding to the protein, relative to monosaccharides, due to increases in their probability of binding. These results have important implications regarding the molecular properties of "so-called" Con A receptors on the surface cells.

Previous Solvent Proton NMR Relaxation Studies of Con A-Saccharide Interactions. A previous study with solvent proton relaxation measurements at two frequencies of solutions of Ca^{2+}–Mn^{2+}–Con A in the presence and absence of mono- and oligosaccharides that bind to the protein was reported by Villafranca & Viola (1974). Their data does not agree with our results in that they found different effects on the solvent proton relaxation rates of Ca^{2+}–Mn^{2+}–Con A solutions for different saccharides. They report a correlation between the percentage change in solvent proton relaxation rate and 50% inhibition concentration for the saccharides tested in a Con

A–dextran precipitation assay. These workers concluded that the more strongly binding saccharides produced the greatest effect on the solvent proton relaxation rates of Ca^{2+}–Mn^{2+}–Con A solutions. We find, however, that all of the saccharides we tested which include melezitose, α-MDM, α-MDG, β-MDG, and maltose which were used by Villafranca and Viola produce the same effect on the solvent proton relaxation rate values of solutions of Ca^{2+}–Mn^{2+}–Con A. We believe that the reason for this discrepancy lies in the concentration of saccharides used in their study which was stated as being tenfold or greater than the K_D value of the saccharides tested. However, we feel that the concentration of saccharides tested was on the order of 10 mM, since their data can then be interpreted as arising from incomplete saturation of the saccharide binding sites on Ca^{2+}–Mn^{2+}–Con A. For example, they report that β-MDG produced only 46% of the effect α-MDM in reducing the observed relaxivity values. (We agree with their value for α-MDM.) We have previously determined that the association constant of β-MDG is approximately 70 M^{-1} (Brewer et al., 1973a,b), and therefore, at a concentration of 10 mM, β-MDG would indeed be binding to essentially only half of the Ca^{2+}–Mn^{2+}–Con A in solution and produce only half of the effect observed when the protein was saturated as it is with α-MDM. Their correlation plot of the percentage change in proton relaxation rate and 50% inhibition for the saccharides in the Con A–dextran inhibition assay support this conclusion. It therefore appears that their data reflect partial binding to Ca^{2+}–Mn^{2+}–Con A for most of the saccharides studied.

Models for Con A Receptors. A variety of complex glycopeptides that bind to Con A with varying affinities have been isolated from macromolecules and the surface of cells and appear to be candidate molecules for "Con A receptors" in these systems. Spiro (1970) has reviewed the structures of two such groups of glycopeptides and classified them as either mannose-*N*-acetylglucosamine types or complex heteropolysaccharide types. Both involve branched chained structures with the former glycopeptides consisting of only mannose and *N*-acetylglucosamine residues in which a large cluster of α-mannosyl residues exists, often α(1→2)-linked or branched to form nonreducing α-mannosyl residues. The glycopeptides of the complex heteropolysaccharide type contain galactose, fucose, and sialic acid in addition to *N*-acetylglucosamine and mannose. Two of the three mannose residues are in the α configuration and are located in the middle region of each of the two branched chains, and possess free 3-, 4-, and 6-hydroxyl groups. These residues appear to be the binding sites to Con A in these molecules.

It has been suggested that these classes of glycopeptides possess binding specificity and, in certain cases, enhanced affinity constants by virtue of interactions with an extended carbohydrate binding site in Con A (cf. Kornfeld & Ferris, 1975; Young & Leon, 1974). However, we suggest that the affinity and specificity of binding of these molecules can be explained by using our single site model for Con A–saccharide interactions. In fact, it is very difficult to imagine a common extended binding site on the protein which could accommodate the variety of structures found for these glycopeptides. Rather, what is a common structural feature among them is the presence of multiple internal and nonreducing terminal α-mannose residues with free 3-, 4-, and 6-hydroxyl groups. The more of these type of residues in the molecule, the greater is the affinity of the glycopeptide for Con A (Ogata et al., 1975). It appears, therefore, that by clustering a group of binding residues (mannose) together, as is found in these glycopeptides, enhanced binding interactions can occur through increased probability of complex formation and recapture with Con A, as discussed above. Indeed, such a mechanism for modulating the affinity of glycopeptides with Con A may be important in terms of the observation that there are different populations of "Con A receptors" found on the surface of normal and transformed cells that are distinguishable by their saturation binding plots (cf. Krug et al., 1973; Huet & Bernadac, 1975). Thus, the present model for Con A–saccharide interactions discussed in this paper may provide insight into the specificity and biological functions of so-called Con A receptors.

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Liposomes as Model Membranes for Ligand–Receptor Interactions: Studies with Choleragen and Glycolipids†

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ABSTRACT: Binding of [^{125}I]choleragen to liposomes containing G_{M1} [galactosyl-*N*-acetylgalactosaminyl(*N*-acetylneuraminyl)galactosylglucosylceramide] had the characteristics previously described for the binding of the toxin to cells and membranes [P. Cuatrecasas (1973) *Biochemistry 12*, 3547]. Binding was rapid, not readily reversible, and saturable. Half-saturation occurred at 10^{-10} M choleragen, and similar concentrations of unlabeled toxin blocked the binding of [^{125}I]choleragen to the liposomes. Binding was highly specific for liposomes containing G_{M1}; only small amounts of toxin bound to liposomes containing the homologous glycolipids G_{D1b} [galactosyl-*N*-acetylgalactosaminyl(*N*-acetylneuraminyl-*N*-acetylneuraminyl)galactosylglucosylceramide], G_{A1} (galactosyl-*N*-acetylgalactosaminygalactosylglucosylceramide), and G_{M2} [*N*-acetylgalactosyl(*N*-acetylneuraminyl)galactosylglucosylceramide.] Choleragen effectively protected the G_{M1} of liposomes from external labeling by sequential treatment with galactose oxidase and NaB^3H_4; incorporation of ^{3}H into the galactose of G_{M1} was reduced by 90%. Liposomal G_{D1b}, G_{A1}, and G_{M2} were protected to a lesser extent. Binding of choleragen also reduced the labeling of the sialic acid residue of liposomal G_{M1} by $NaIO_4$ and NaB^3H_4. These results are similar to those reported for G_{M1} in intact cells [J. Moss et al. (1977) *Biochemistry 16*, 1876]. Thus, the interaction of choleragen with G_{M1} incorporated into lipid model membranes mimicked the characteristics and specificity noted with biological membranes. Liposomes appear to be useful as model membranes to explore the interaction of ligands with glycolipids incorporated into the liposomal membranes.

Liposomes (lipid model membranes) have been extremely useful in studying the interactions of antibodies with glycolipid antigens (Kinsky, 1972; Alving, 1977). Many of the properties of the antibody–antigen reaction correspond to what is observed with biological membranes. Liposomes containing various glycolipids also bind lectins (Surolia et al., 1975; Boldt et al., 1977), hormones (Maggio et al., 1977; Aloj et al., 1977; Pacuszka et al., 1978b), and toxins (Moss et al., 1976, 1977c). In none of these studies, however, has the interaction of the ligands with the liposomes been examined in detail to determine their suitability as model membranes for ligand–glycolipid interactions.

The monosialoganglioside G_{M1}[1] has been implicated as the membrane receptor for choleragen (Cuatrecasas, 1973a,b; Holmgren et al., 1973; King & van Heyningen, 1973), and its binding to cells and membranes has been well characterized (Cuatrecasas, 1973a–c). The interaction of choleragen with

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[1] Abbreviations used: G_{M1}, Galβ1→3GalNAcβ1→4Gal[3←2αAcNeu]β1→4Glcβ1→ceramide; G_{M2}, GalNAcβ1→4Gal[3←2αAcNeu]β1→4Glcβ1→ceramide; G_{M3}, AcNeuα2→3Galβ1→4Glcβ1→ceramide; G_{D1a}, AcNeuα2→3Galβ1→3GalNAcβ1→4Gal[3←2αAcNeu]β1→4Glcβ1→ceramide; G_{D1b}, Galβ1→3GalNAcβ1→4Gal[3←2αAcNeu8←2αAcNeu]β1→4Glcβ1→ceramide; G_{A1}, Galβ1→3GalNAcβ1→4Galβ1→4Glcβ1→ceramide; GL-4, GalNAcβ1→4Galα1→3Galβ1→4Glcβ1→ceramide; AcNeu, *N*-acetylneuraminic acid; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid.