Hassing, G. S., and Goldstein, I. J. (1970), *Eur. J. Biochem. 16*, 549.
Jaffé, W. G., and Palozzo, A. (1971), *Acta Cient. Venez. 22*, 102.
Johnson, C. K. (1965), Oak Ridge Technological Manual, Oak Ridge, Tenn., Oak Ridge National Laboratory 3794.
Noonan, K. D., and Burger, M. M. (1973), Abstracts of the Ninth International Congress of Biochemistry, Stockholm, July 1–7, 1973, p 275.
Poretz, R. D., and Goldstein, I. J. (1970), *Biochemistry 9*, 2890.
Sharon, N., and Lis, H. (1972), *Science 177*, 949.
Sly, D. A. (1972), Ph.D. Dissertation, University of Minnesota.

Interactions of Saccharides with Concanavalin A. Mechanism of Binding of α- and β-Methyl D-Glucopyranoside to Concanavalin A as Determined by ^{13}C Nuclear Magnetic Resonance†

C. F. Brewer,*,‡ H. Sternlicht,§ D. M. Marcus,¶ and A. P. Grollman

ABSTRACT: Binding of α- and β-methyl D-glucopyranoside (uniformly labeled with 14% ^{13}C) to concanavalin A was studied by pulsed Fourier transform carbon magnetic resonance techniques. The spin-lattice relaxation times (T_1) for the carbon resonances of the two glycosides were measured in the presence and absence of the zinc and manganese derivatives of the protein. T_1 values for the ring carbons of both sugars were uniformly shortened when bound to zinc concanavalin A and selectively shortened when bound to manganese concanavalin A. The results indicate that the paramagnetic managanese ion in concanavalin A contributes to the relaxation of the carbon atoms of the bound sugars. The distance between each carbon atom of the bound sugars and the manganese ion was calculated from the paramagnetic contribution of manganese to the T_1 of the sugar carbons. These measurements establish the three-dimensional orientation of both anomers relative to the transition metal site in the protein. Both sugars appear to bind in different orientations while remaining in the C-1 chair conformation with their nonreducing ends closest to the manganese ion at a mean distance of 10 Å. The distance separating the transition metal and sugar binding site in the protein, as determined from these measurements in solution, differs from the value recently proposed on the basis of X-ray diffraction studies. The different binding orientations of α- and β-methyl D-glucopyranoside account for the difference in binding constants of the two sugars and the relative affinities of their derivatives for concanavalin A.

Concanavalin A (Con A),[1] a protein isolated from the Jack bean (*Canavalia ensiformis*) (Sumner and Howell, 1936), is a member of the lectin class of plant proteins. Interest in Con A stems from its unusual effects on animal cells (*cf.* Sharon and Lis, 1972). The protein agglutinates cells transformed by oncogenic viruses (Inbar and Sachs, 1969), inhibits growth of malignant cells in experimental animals (Shoham *et al.*, 1970), and exhibits mitogenic activity (Powell and Leon, 1970). Fibroblasts transformed by oncogenic viruses *in vitro* are restored to normal cell density by exposure to trypsin-treated Con A (Burger and Noonan, 1970).

The biological effects of Con A appear to be related to its sugar binding properties. Con A binds monosaccharides with the D-mannopyranoside configuration at the 3, 4, and 6 positions (Goldstein *et al.*, 1965b), and interacts preferentially with the α anomers of these sugars. The ability of Con A to precipitate certain polysaccharides has served as a model for antibody–antigen reactions (Goldstein *et al.*, 1965a).

Between pH 3.5 and 5.6, Con A exists as a dimer with a molecular weight of 54,000; at higher pH, tetramers form (Kalb and Lustig, 1968; McKenzie *et al.*, 1972). Each monomeric unit has one site (S_1) that binds transition metal ions and another site (S_2) that binds calcium ions. Evidence has been presented suggesting that both the S_1 and S_2 sites in the protein must be occupied for saccharide binding activity to occur (Kalb and Levitzki, 1968). Derivatives of Con A in which Zn^{2+}, Co^{2+}, or Mn^{2+} ions occupy the S_1 site possess equal abilities to bind sugars. The S_1 site has been shown by X-ray diffraction studies to be located at the base of a cavity extending to the surface of each monomer (Weinzierl and Kalb, 1971). The primary sequence and electron density map of the protein at 2-Å resolution (Edelman *et al.*, 1972) and at 2.4-Å resolution (Hardman and Ainsworth, 1972) have recently been reported.

† From the Departments of Pharmacology, Medicine, Microbiology and Immunology, and the Division of Biological Sciences, Department of Molecular Biology, Albert Einstein College of Medicine, Bronx, New York 10461, and Bell Laboratories, Murray Hill, New Jersey 07974. *Received May 8, 1973.* That portion of the investigation carried out at Albert Einstein College of Medicine was supported in part by the U. S. Public Health Service, National Institutes of Health Grant AI-05336, and Grant IC-61D from the American Cancer Society.

‡ Recipient of a National Institutes of Health Postdoctoral Traineeship (U. S. Public Health Service Grant GM-00065) in the Department of Pharmacology, Albert Einstein College of Medicine.

¶ Career Scientist of the Health Research Council of the City of New York. Paper I in this series is Brewer *et al.* (1973).

§ Member of Technical Staff, Bell Laboratories.

[1] Abbreviations used are: Con A, concanavalin A; α- and β-MeGlc, α- and β-methyl D-glucopyranoside.

Previous nmr studies of sugar binding to proteins have been limited to mono- and oligosaccharide binding to lysozyme (*cf.* Dahlquist and Raftery, 1969; Millett and Raftery (1972)) and were experimentally restricted to measurements of a few resolvable ^{19}F and ^{1}H resonances of the sugars under binding conditions. Affinity constants, kinetic parameters of binding, and the number of binding sites on the protein were determined, but only qualitative differences in the binding orientation of anomeric sugars could be inferred from chemical-shift data. In contrast, ^{13}C nmr can provide dynamic and structural information from essentially all of the carbons of the bound sugar.

We have briefly reported a ^{13}C nmr study of the binding of α-methyl D-glucopyranoside to Con A (Brewer *et al.*, 1973). The present paper details the mechanism of binding of α- and β-methyl D-glucopyranoside, uniformly labeled with 14% ^{13}C, to Con A and to transition metal derivatives of the protein as studied by pulsed Fourier transform ^{13}C nmr techniques. The effects of the manganese and zinc derivatives of Con A on the ^{13}C spin–lattice relaxation times of the carbons of both sugars permit calculation of the three-dimensional orientation of the bound sugars relative to the transition metal ion site in the protein. Different binding orientations and interactions were found for each anomer which may account for the difference in the affinity constants of these glycosides and explain the relative affinity constants of many of their derivatives. The mean distance between the binding site for both sugars and the Mn^{2+} ion was calculated to be 10 Å, which contrasts with the values of 20 and 23 Å proposed by Edelman *et al.* (1972) and Hardman and Ainsworth (1972), respectively, from X-ray diffraction studies of crystalline complexes of Con A with saccharide derivatives.

Materials and Methods

Synthesis of ^{13}C-Labeled Sugars. α- and β-MeGlc uniformly labeled with 13.8% ^{13}C were synthesized from ^{13}C 13.8% uniformly labeled D-glucose (Lot No. C-909, Merck Sharp and Dohme). The method consisted of refluxing ^{13}C-labeled D-glucose in methanol in the presence of a cation exchange resin (Bartlett and Sheppard, 1969). The products, ^{13}C-labeled α- and β-MeGlc, were separated on an anion exchange column (Austin *et al.*, 1963); elution was monitored by gas–liquid chromatographic analysis of the trimethylsilyl derivatives of the eluate. ^{13}C-Labeled α-MeGlc was crystallized from ethanol–ether to a constant melting point, 167–169° (lit. mp 166° (Conchie *et al.*, 1957), recrystallized from aqueous ethanol). ^{13}C-Labeled β-MeGlc was also recrystallized from ethanol–ether to a constant melting point, 111–112° (lit. mp 110° (Conchie *et al.*, 1957), recrystallized from aqueous ethanol). ^{13}C nuclear magnetic resonance (nmr) spectra of both sugars were identical with those previously reported (Perlin *et al.*, 1970; Dorman and Roberts, 1970).

Preparation of Con A Derivatives. Native Con A, purchased from Miles-Yeda, contained a mixture of Mn^{2+}, Mg^{2+}, Zn^{2+}, and Co^{2+} ions. Zinc Con A (Zn-Con A) and cobalt Con A (Co-Con A) were also obtained from Miles-Yeda. The preparation of Mn-Con A used in this study has been reported elsewhere (Koenig *et al.*, 1973). All metal derivatives of Con A displayed a single band following polyacrylamide gel electrophoresis at pH 4.3. Atomic absorption analysis confirmed the presence of at least 90% of the appropriate transition metal in each protein derivative and 2 equiv of transition metal ion per protein dimer. Atomic absorption analysis of these same protein solutions indicated the presence of ~18% of the theoretical 2 equiv of Ca^{2+} ions per protein dimer. (In our nmr studies, addition of Ca^{2+} ion did not affect the sugar binding activity of Con A (details will be reported elsewhere).) Native Con A and the transition metal derivatives were equally active in agglutinating sheep erythrocytes at ambient temperature (Marcus and Grollman, 1966). All metal derivatives of Con A showed the same kinetics for sugar binding C. F. Brewer *et al.*, manuscript in preparation).

Nmr Measurements. A description of the materials and methods used in the nmr measurements has been published elsewhere (Brewer *et al.*, 1973). Spin–lattice relaxation times (T_1) were measured using the 180°–Δt–90° two pulse sequence (Vold *et al.*, 1968) and varying the time interval, Δt, between the pulses. Transverse relaxation times (T_2) were determined from $\Delta\nu$, the line width in hertz at half-height of the appropriate resonance using the relationship $\Delta\nu = (\pi T_2)^{-1}$.

Relaxation Theory

Transverse, T_{2o}, and spin–lattice, T_{1o}, relaxation times of the ^{13}C carbons of free sugars in solution are determined primarily by fluctuating magnetic dipolar interactions between the spins of the ^{13}C carbons and their directly bonded hydrogen atom(s). In the presence of a diamagnetic protein that binds the sugars, these same dipolar interactions determine the relaxation times of the carbons of the bound sugar. The differences between the relaxation times of the free and bound sugars are related to the difference in molecular rotational correlation time, τ_r, of the two states of the sugar. The slower tumbling time of the sugar–protein complex increases the spectral density of magnetic fluctuations (Abragam, 1961) at the ^{13}C Larmor frequencies, thereby facilitating spin transitions between the two ^{13}C spin energy states.

The experimentally observed transverse and spin–lattice relaxation times, T_2 and T_1, respectively, of the carbon spins of the sugars in the presence of the protein are given by eq 1a and 1b, where T_{2o} and T_{1o} are the transverse and spin–

$$T_2^{-1} = T_{2o}^{-1} + f(T_{2m} + \tau_m)^{-1} \quad (1a)$$

$$T_1^{-1} = T_{1o}^{-1} + f(T_{1m} + \tau_m)^{-1} \quad (1b)$$

lattice relaxation times, respectively, of the ^{13}C carbons of the sugar in the absence of protein, f is the ratio of bound to total sugar in solution, T_{2m} and T_{1m} are the transverse and spin–lattice relaxation times, respectively, of the ^{13}C carbons of the sugar bound to the protein, and τ_m is the residence time of the sugars on the protein. Equations 1a and 1b assume that there is an excess of sugar in solution relative to the protein concentration ($f \ll 1$) and that the differences in chemical shift between free and bound sugars are negligible (Swift and Connick, 1962). Ring carbons of the sugars are expected to have a single value for τ_m because they bind as a rigid unit. T_{2m}, T_{1m}, and τ_m are determined from studies of the temperature dependence of T_2 and T_1 as a function of f. τ_m generally decreases with increasing temperature (Abragam, 1961); T_{2m} generally increases with increasing temperature (Abragam, 1961). Two processes may contribute to T_{2m} and T_{1m}: (i) diamagnetic relaxation that arises from the magnetic dipolar interaction between the nuclear spin(s) of the ^{13}C carbon(s) and its neighboring hydrogens and (ii) paramagnetic relaxation that arises from the magnetic dipolar interaction between the ^{13}C spin(s) and the unpaired electron spins of the paramagnetic ion bound to the protein. Paramagnetic relaxation must be considered for the Mn- and

Co-Con A derivatives, while only diamagnetic relaxation exists for Zn-Con A.

For Zn-Con A, T_{2m} and T_{1m} of the bound sugars are given by eq 2a and 2b (Abragam, 1961), where n is the number of

$$T_{2m}^{-1} = T_{2m,d}^{-1} = (4n/15)Kr_{CH}^{-6}\tau_r\{1 + 0.25[1 + (\omega_S - \omega_I)^2\tau_r^2]^{-1} + 0.75[1 + \omega_I^2\tau_r^2]^{-1} + 1.5[1 + \omega_S^2\tau_r^2]^{-1} + 1.5[1 + (\omega_S + \omega_I)^2\tau_r^2]^{-1}\} \quad (2a)$$

$$T_{1m}^{-1} = T_{1m,d}^{-1} = (4n/15)Kr_{CH}^{-6}\tau_r\{0.5[1 + (\omega_S - \omega_I)^2\tau_r^2]^{-1} + 1.5[1 + \omega_I^2\tau_r^2]^{-1} + 3[1 + (\omega_S + \omega_I)^2\tau_r^2]^{-1}\} \quad (2b)$$

directly bonded protons and $K = \hbar^2\gamma_S^2\gamma_I^2S(S + 1)$, where $\hbar$ is Planck's constant divided by 2π, γ_S and γ_I are the proton and carbon gyromagnetic constants, respectively, and S is the ^1H spin value of 0.5. The covalent carbon–proton distance is denoted as r_{CH}. τ_r is the rotational correlation time of the sugar–protein complex and decreases with increasing temperature. ω_S and ω_I are the Larmor precession frequencies in radians per second of the ^1H and ^{13}C nuclear spins, respectively. For the ring carbons, $n = 1$; for C-6, $n = 2$. The assignments for n are made with the assumption that only directly bonded hydrogens contribute to the relaxation times of the ^{13}C carbons. Intermolecular dipole–dipole relaxations due to protein hydrogens were not deemed an important source of relaxation because of the r^{-6} distance factor in eq 2a and 2b. The contribution of directly bonded ^{13}C carbons to the observed carbon relaxation is also unimportant because of the low degree of ^{13}C enrichment, the small gyromagnetic constant of carbon relative to hydrogen, and large carbon–carbon bond distance relative to that between carbon and hydrogen atoms.

In the case of Mn-Con A or Co-Con A, T_{2m}^{-1} and T_{1m}^{-1} of the bound sugars are given by

$$T_{2m}^{-1} = T_{2m,d}^{-1} + T_{2p}^{-1} \quad (3a)$$

$$T_{1m}^{-1} = T_{1m,d}^{-1} + T_{1p}^{-1} \quad (3b)$$

where $T_{2m,d}^{-1}$ and $T_{1m,d}^{-1}$ are the diamagnetic contributions to the transverse and spin–lattice relaxation rates, respectively, of the ^{13}C carbons of bound sugars (eq 2a and 2b), and T_{2p}^{-1} and T_{1p}^{-1} are the paramagnetic contributions of the metal ion to the respective relaxation rates of the bound sugar carbons. Assuming that $T_{2m,d}$ and $T_{1m,d}$ of Mn- and Co-Con A are the same as those for Zn-Con A, T_{2p}^{-1} and T_{1p}^{-1} are obtained by subtracting the reciprocal relaxation times of the sugars bound to Zn-Con A from the reciprocal relaxation times of the sugars bound to Co- or Mn-Con A (eq 3). (In our study T_{2p} could not be determined because of the relatively long τ_m exchange times.) At 23.4 kG T_{1p}, the paramagnetic contribution to T_{1m}, is given by eq 4 (Abragam, 1961; Shulman *et al.*, 1965), where g denotes the electronic "g" factor, β represents the Bohr magneton, S is the electron spin quantum number of the paramagnetic ion, r_{CM} is the metal ion–carbon internuclear distance, and τ_e is the electron–nuclear correlation time for dipolar interactions at 23.4 kG. In the case of Mn-Con A, the values $g = 2.0$ and $S = 5/2$ provide reasonable approximations (Rubenstein *et al.*, 1971; Reed and Cohn, 1970). The electron–nuclear correlation time, τ_e is given by eq 5, where τ_s is the

$$T_{1p}^{-1} = (2/5)g^2\beta^2\gamma_I^2S(S + 1)r_{CM}^{-6}\tau_e(1 + \omega_I^2\tau_e^2)^{-1} \quad (4)$$

$$\tau_e^{-1} = \tau_r^{-1} + \tau_m^{-1} + \tau_s^{-1} \quad (5)$$

electron spin–lattice relaxation time at 23.4 kG. For Mn-Con A τ_e was found (Koenig *et al.*, 1973) to be dominated by τ_r, the rotation correlation time of the protein in solution, such that $\tau_e = \tau_r$ (see below). The Fermi-contact contribution (Bloembergen and Morgan, 1961) to the values for T_{1p} was neglected. This is valid when no unpaired spin density is delocalized from the paramagnetic ion into the sugar ring as found in the case of sugars bound to Con A. The expressions for T_{1p}^{-1} follow directly from eq 2b with $n = 1$, γ_S replaced by $g\beta\hbar^{-1}$, r_{CH} replaced by r_{CM}, τ_r replaced by τ_e and ω_S set equal to the electron Larmor frequency at 23.4 kG. The simplified expression (eq 4) results since the electron Larmor frequency is several orders of magnitude larger than that of the ^{13}C carbon spin.

The above dipolar expressions for $T_{2m,d}$, $T_{1m,d}$, and T_{1p} involve only one rotational correlation time, τ_r, and are written independent of orientational factors. This is correct for isotropic rotational Brownian motion; however, Con A, a dimer at pH 5.60, should be treated hydrodynamically as an axially symmetric ellipsoid with semimajor axes a, b, and c equal to 42, 20, and 20 Å, respectively (Edelman *et al.*, 1972). The rotational diffusion motions of ellipsoids are anisotropic (Perrin, 1936; Woessner, 1962). In contrast to the isotropic case, the relaxation times T_{2m} and T_{1m} of sugars bound to Con A would be expected to depend on the orientations of r_{CH} and r_{CM}, relative to the a axis (Woessner, 1962). In addition, the nmr relaxation expressions depend on more than one rotational diffusion time. The modified relaxation expressions are slowly varying functions of the orientation of the r_{CH} or r_{CM} vectors relative to the a semimajor axis.

According to Woessner, the calculated spin relaxation times of any carbon of the bound sugar based on a common isotropic correlation time for all carbons (eq 2a, 2b, and 4) could be in error by 50% depending on the orientation factors and differences in the rotation diffusion times about the ellipsoidal axis. The anisotropic rotational motion of the protein could therefore cause an absolute error of ~10% in the calculated carbon–metal ion distances as obtained from the isotropic expression. However, the relative error in the calculated distances between the carbons of the sugar and the metal ion could still be small (<5%). For example, in cases where the carbon–metal ion distances are sufficiently large, all r_{CM} vectors make approximately the same angle with the semimajor axes, a. A common isotropic tumbling correlation time for the protein–sugar complex could therefore be used to calculate the relative distances of the sugar carbons to the metal ion with an error of less than 5%.

Results

Effect of Protein Binding on the ^{13}C Resonances of the Sugars. The proton-decoupled ^{13}C nmr spectrum of ^{13}C-enriched α-MeGlc is shown in the upper part of Figure 1. The carbon resonance assignments are listed above each peak. Small peaks on either side of major peaks result from ^{13}C–^{13}C coupling between directly bonded ^{13}C carbons.

The ^{13}C spectrum of the α anomer in the presence of Zn-Con A (Figure 1A) or any of the other Con A derivatives shows line broadening which is reduced if an excess of

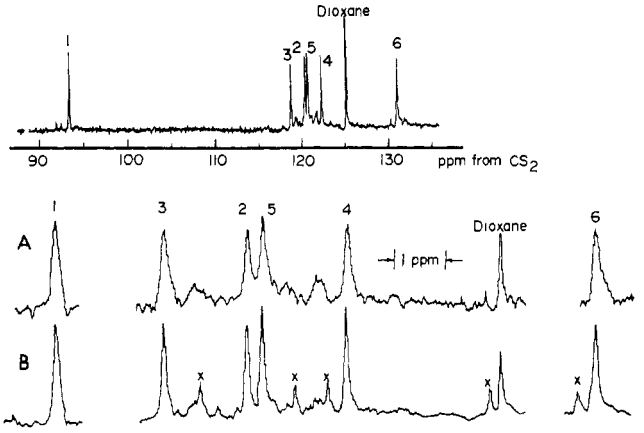

FIGURE 1: Proton-decoupled ^{13}C nmr spectra of ^{13}C-enriched α-methyl D-glucopyranoside at pH 5.6 and 25° and (A) in the presence of Zn-Con A; (B) in the presence of Zn-Con A and α-methyl D-mannopyranoside. The concentrations used were: 1.25×10^{-2} M ^{13}C-enriched α-methyl D-glucopyranoside, 8.7×10^{-4} M Zn-Con A, and 4×10^{-2} M α-methyl D-mannopyranoside. Spectra A and B are shown on an expanded scale. The small peaks (×) in spectrum B are those of α-methyl D-mannopyranoside.

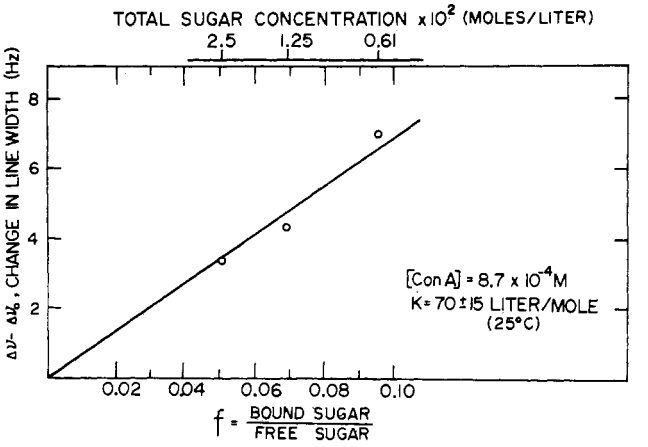

FIGURE 2: Plot of the change in the C-6 line width of ^{13}C-enriched β-methyl D-glucopyranoside in the presence of 8.7×10^{-4} M "native" Con A as a function of f, the ratio of bound to unbound sugar in solution. The total sugar concentration present ranged from 0.61×10^{-2} to 2.5×10^{-2} M. The affinity constant, K_a, is based on two independent sugar binding sites per Con A dimer (molar 54,000).

α-methyl D-mannopyranoside, a competitive binding sugar (Goldstein *et al.*, 1965b), is added to the solution (Figure 1B). The line broadening in Figure 1A is, therefore, due to specific protein–saccharide binding and not due to factors such as viscosity changes in the solution. Line broadening in the spectrum of the β anomer also occurs in the presence of the protein. No chemical shifts of the carbon resonance positions for either sugar were observed upon addition of protein to solution, or as a function of temperature.

The integrated signal intensity of the proton decoupled ^{13}C resonances of the sugar carbons in the presence of the protein was reduced by approximately a factor of 2 relative to the spectrum of the free sugars. These results indicate a loss of the nuclear Overhauser enhancement (NOE) for the sugar carbon resonances upon binding to the protein. The NOE was regained upon addition of an excess of α-methyl D-mannopyranoside to the solution.

The change in line widths at half-peak height of the carbon resonances of the α anomer showed a linear relationship to the total sugar concentration (6.1–25 mM) in the presence of a constant amount of protein (0.49–0.87 mM). A similar plot for the β anomer was nonlinear, indicating incomplete binding to the protein. The affinity constant for the β anomer was calculated to be 70 ± 15 at 25° from these data. This value was used to determine f, the fraction of bound to total sugar in solution in the presence of protein. Figure 2 shows a plot of the change in line width of the resonance of C-6 of the β anomer as a function of f. The linear reationship (Figure 2) demonstrates that the observed line broadening of the β anomer also results from specific binding to the protein.

Effects of Temperature on the ^{13}C Resonances of the Sugars in the Presence of the Con A Derivatives. Temperature dependence (between 4 and 45°) of the line widths of C-1 and -6 resonances for both sugars in the presence of native Con A at pH 5.60 is shown in Figure 3. Line broadening, represented as log $[f/\pi(\Delta\nu - \Delta\nu_0)]$, is plotted against the reciprocal absolute temperature, where $\Delta\nu$ and $\Delta\nu_0$ are the line widths at half-peak height of the ^{13}C sugar resonances in the presence and absence of protein, respectively. $f/\pi(\Delta\nu - \Delta\nu_0)$ is equal to $T_{2m} + \tau_m$ (eq 1a). Between 4 and 25°, the line widths for the carbon resonances of the α anomer were nearly the same and broadened with increasing temperature. In this temperature region, the α anomer exchanges slowly on the nmr time scale between free and protein-bound environments, and the line-broadening effects are dominated by the residence time of the sugar on the protein ($\tau_m > T_{2m}$). Above 25°, the line widths of the ring carbons of the α anomer begin to narrow, indicating intermediate to fast exchange between free and protein-bound environments (*i.e.*, $T_{2m} > \tau_m$), since T_{2m} is known to increase with increasing temperature (Abragam, 1961). The line width of C-6, however, continues to increase above 25° which indicates that C-6 of the α anomer was still undergoing slow exchange with the protein ($\tau_m > T_{2m}$). This result is consistent with T_{2m} of

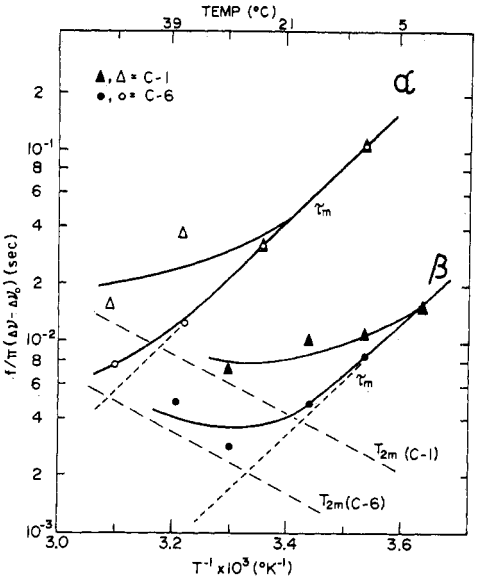

FIGURE 3: Plot of the line broadening of the C-1 and C-6 resonances of ^{13}C-enriched α- and β-methyl D-glucopyranoside at pH 5.60 in the presence of "native" Con A as a function of temperature. The line broadening effects are expressed as log $[f/\pi(\Delta\nu - \Delta\nu_0)]$ where $\Delta\nu - \Delta\nu_0$ denotes the change in line width in the presence of protein and f denotes the ratio of bound to unbound sugar. The total sugar concentration varied from 0.61×10^{-2} to 5×10^{-2} M. The Con A concentration varied from 4.8×10^{-4} to 8.7×10^{-4} M. The measurements at 50° were complicated by the instability of the protein at this temperature.

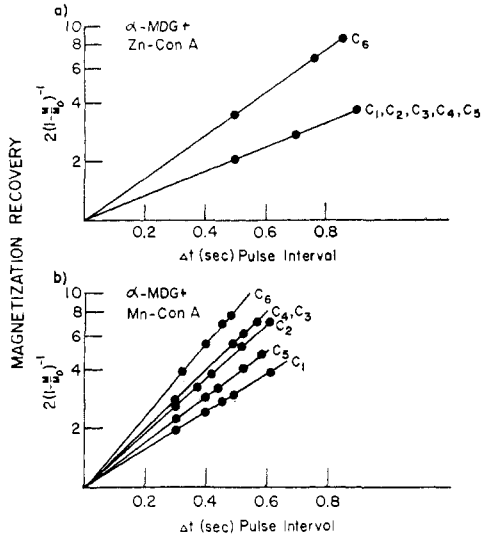

FIGURE 4: Plot of the ^{13}C magnetization recovery *vs.* pulse interval, Δt, for 1.25×10^{-2} M ^{13}C-labeled α-methyl D-glucopyranoside in the presence of (a) 8.7×10^{-4} M Zn-Con A and (b) 8.7×10^{-4} M Mn-Con A. $2[1 - (M/M_0)]^{-1}$ is equal to 2.718 when $\Delta t = T_1$. M_0 is the full magnetization value of a given carbon after $6T_1$ times (complete relaxation) following the 180° pulse; M denotes the net magnetization value (represented by the observed signal intensity) after the pulse interval, Δt, following the 180° pulse.

C-6 being shorter than T_{2m} of the ring carbons, since C-6 possesses an extra proton. At 25°, the α anomer was determined to have a $\tau_m = 2.5 \times 10^{-2}$ sec. Similar findings were obtained for the transition metal derivatives of the protein.

The results for the β anomer (Figure 3) are similar to those observed for the α anomer, but differ in the magnitude of τ_m. At 25°, τ_m for the β anomer was 2.5×10^{-3} sec. This value was determined from a temperature study of the line width of C-6 (Figure 3), since T_{2m} influences this carbon's line width less at 25° than for ring carbons with single bonded protons (Figure 3). The T_{2m} value of the ring carbons for both sugars bound to native Con A at 25° was estimated to be $6 \pm 2 \times 10^{-3}$ sec. T_{2m} of C-6 for both sugars was half of this value due to the presence of an extra proton. Similar data between 4 and 25° were obtained for the sugars in the presence of the various transition metal derivatives of Con A.

T_1 Measurements of the Sugars in the Presence of Con A. T_{2m} could not be accurately determined from the effects of protein binding on the transverse relaxation times of the ^{13}C sugar carbons at 25° due to the relatively large contribution of τ_m (Figure 3) to the observed T_2. ^{13}C spin–lattice relaxation times (T_1) of the sugar carbons in the presence of protein were therefore determined. Since $T_{1m} \geq T_{2m}$, depending on the rotational correlation time of the sugar–protein complex (Doddrell *et al.*, 1972), contributions of τ_m to the observed T_1 can be relatively minor.

T_1 values for each of the carbon resonances of ^{13}C-enriched α-methyl D-glucopyranoside can be determined simultaneously by the 180°–Δt–90° pulse sequence method (Vold *et al.*, 1968). The same is true for the β anomer, except that carbon resonances 3 and 5 overlap. A typical plot of the magnetization recovery of the ^{13}C carbons of the α anomer in the presence of Zn- and Mn-Con A as a function of the pulse interval, Δt, is shown in Figures 4a and 4b, respectively. Each of the ^{13}C carbons of the sugar experiences a reduced, as well as a unique, relaxation time in the presence of Mn-Con A due to the presence of the paramagnetic Mn^{2+} ion. No significant difference in the observed T_1 of the carbons of either sugar was observed in the presence of Zn- or Co-Con A. Small corrections for τ_m were made (eq 1b) in calculating the T_{1m} data.

TABLE 1: Spin–Lattice Relaxation Time of ^{13}C Carbons of α-Methyl D-Glucopyranoside in the Presence of the Zinc, Cobalt, and Manganese Transition Metal Derivatives of Con A at 25° ($\tau_m = 0.025 \pm 0.005$ sec; Concentration of Protein, 8.7×10^{-4} M).

Con A Transition Metal Derivative	Carbon	T_1 (sec)[a] Free sugar	f^b = 0.071	f^c = 0.14	T_{1m} (sec)		T_{1p}[d,e] (sec)
Free sugar (no protein)	1	1.09					
	2	1.06					
	3	1.03					
	4	1.03					
	5	1.07					
	6	0.60					
Zn-Con A	1		0.86	0.71	0.26		
	2		0.82	0.72	0.26		
	3		0.82	0.72	0.29	0.28[d]	
	4		0.84	0.72	0.30		
	5		0.83	0.75	0.28		
	6		0.45	0.40	0.12		
Co-Con A	1		0.84		0.24		
	2		0.84		0.26		
	3		0.85		0.32	0.27[d]	
	4		0.85		0.32		
	5		0.82		0.22		
	6		0.47		0.13		
Mn-Con A	1		0.56	0.45	0.069		0.091
	2		0.46	0.32	0.036		0.040
	3		0.40	0.29	0.026		0.028
	4		0.40	0.29	0.026		0.028
	5		0.49	0.38	0.048		0.057
	6		0.33	0.24	0.029		0.038

[a] T_1 times were determined accurately to ±0.03 sec. [b] Total sugar concentration was 2.5×10^{-2} M. [c] Total sugar concentration was 1.25×10^{-2} M. [d] An average T_{1m} of *ca.* 0.28 sec for the ring carbons of sugars bound to Zn- and Co-Con A was used in calculating T_{1p}. The differences in the individual T_{1m} values reflect experimental errors in determining T_1 values. [e] The estimated error in T_{1p} is *ca.* ±15% for the ring carbons and *ca.* ±25% for C-6.

Measurements of the Affinity Constants of the Sugars. Determination of the affinity constants, K_a, for binding of α- and β-MeGlc to Con A at 25° has been described elsewhere (Brewer *et al.*, 1973). The K_a values found for the α and β anomers were 1700 ± 300 M^{-1} and 70 ± 15 M^{-1}, respectively. The ratio of the affinity constants for the two anomers agrees well with the literature value of 28 determined at 2° from hapten-inhibition experiments (So and Goldstein, 1968). The nmr calculated value for the K_a of the β anomer at 2° also agrees well with the literature value (So and Goldstein, 1968).

Discussion

Binding of α- and β-MeGlc to Zn-Con A. The observed spin–lattice relaxation times of ^{13}C ring carbons for both

TABLE II: Spin–Lattice Relaxation Time of the ^{13}C Carbons of β-Methyl D-Glucopyranoside in the Presence of the Zinc, Cobalt, and Manganese Transition Metal Derivative of Con A at 25° ($\tau_m = 2.5 \times 10^{-3}$ sec; Concentration of Protein, 8.7×10^{-4} M).

Con A Transition Metal Derivative	Carbon	T_1[a] (sec) Free Sugar	T_1[a] (sec) f = 0.049[b]	T_1[a] (sec) f = 0.073[c]	T_{1m} (sec)	T_{1p}[d,e] (sec)
Free sugar (no protein)	1	1.08				
	2	1.05				
	4	1.06				
	3, 5	1.06				
	6	0.56				
Zn-Con A	1		0.88		0.23	0.26[d]
	2		0.85		0.22	
	4		0.86		0.22	
	3, 5		0.81			
	6		0.43		0.09	0.11[d]
Mn-Con A	1		0.63	0.56	0.078	0.11
	2		0.55	0.48	0.058	0.075
	4		0.37	0.36	0.030	0.034
	3, 5[f]		0.50	0.42	0.045	0.054
	6		0.36	0.34	0.053	0.10

[a] T_1 times were determined accurately to ±0.03 sec. [b] Total sugar concentration was 2.5×10^{-2} M. [c] Total sugar concentration was 1.25×10^{-2} M. f was calculated using an affinity constant K_1 equal to 70 ± 15 l. mol^{-1} at 25°. [d] An average value for T_{1m} of the β anomer bound to Zn-Con A is based on a $K_1 = 70 \pm 15.1$ mol^{-1}. An average of *ca.* 0.26 sec for the ring carbons and 0.11 sec for C-6 based on the combined data of α- and β-MeGlc was used in calculating T_{1p} (see Table I). [e] The estimated error in T_{1p} is slightly larger than that given for the α anomer (Table I). [f] The C-3 and C-5 resonances are unresolved. T_1 and the common T_{1m} and T_{1p} values given above represent apparent values and are subject to appreciable uncertainty.

TABLE III: Experimental (Exp) and Computer Fitted (CF) Distances between the Manganese Ion and Carbons of α- and β-MeGlc to Mn-Con A at 25°.

	Carbon	T_{1p} (sec)	Distance (Å) Exp[a]	Distance (Å) CF
α-[^{13}C]MeGlc	1	0.091	11.7	11.5
	2	0.040	10.2	10.4
	3	0.028	9.6	9.8
	4	0.028	9.6	9.4
	5	0.057	10.8	10.6
	6	0.038	10.1	10.4
β-[^{13}C]MeGlc	1	0.11	12.0	12.0
	2	0.075	11.2	11.4
	3[b]	0.054[b]	10.6	10.0
	4	0.034	9.9	10.2
	5[b]	0.054[b]	10.6	10.9
	6	0.10	11.8	11.5

[a] τ_r is 8×10^{-8} sec at 25°. The estimated error in r (Å) for this τ_r value is ±0.3 Å. [b] These resonances are unresolved at 23.4 kG. T_{1p} is an apparent average value for the relaxation time.

α- and β-MeGlc were uniformly shortened in the presence of Zn-Con A (Tables I and II). T_{1m} values for the bound sugars were calculated from eq 1b; τ_m values for α- and β-MeGlc at 25° were 2.5×10^{-2} and 2.5×10^{-3} sec, respectively, as determined from temperature studies of the observed linewidth behavior of the sugar carbons in the presence of the protein (Figure 3). (A discussion of the kinetics of binding of α- and β-MeGlc is presented elsewhere (Brewer *et al.*, 1972).) The T_{1m} value obtained for C-6 of both sugars is shorter by a factor of 2 than those of the ring carbons, suggesting that the principal mechanism for spin–lattice relaxation of the ^{13}C carbons in the bound sugar is intramolecular ^{13}C–^{1}H dipolar relaxation involving directly bonded hydrogen(s). T_{1m} values for the ring carbons of the sugars bound to Zn-Con A were observed to be essentially the same despite the expected anisotropic tumbling motion of the protein and can be assigned an "effective" common isotropic rotation correlation time (τ_r). The α anomer has a high affinity constant and binds all of the protein in solution allowing an accurate determination of f. Thus, precise calculation of τ_r was obtained from the T_{1m} data of the α anomer bound to Zn- or Co-Con A.

Two possible values for τ_r satisfy eq 2b: 8×10^{-8} and 2×10^{-9} sec. The longer value for τ_r was chosen for reasons that have been discussed elsewhere (Brewer *et al.*, 1973). The τ_r value for the sugar–protein complex suggests that the sugars are tightly bound to the protein and tumble with a time characteristic of the tumbling time of the protein.

Binding of α-MeGlc to Co-Con A. T_1 values of the α anomer in the presence of paramagnetic Co-Con A were the same as those observed in the presence of Zn-Con A (Table I). This result was expected since the electron spin–lattice relaxation time, τ_s, of the cobalt ion bound to protein should be very short at 23.4 kG (Eisinger *et al.*, 1962; S. Koenig, R. Brown, and C. F. Brewer, unpublished data) and thus $\tau_c = \tau_s$ for the sugar bound to Co-Con A. With a short τ_s, Co-Con A is not an effective source for electron–nuclear relaxation and would only contribute to nuclear relaxation of the sugar carbons if the sugar was bound to or very close to the ion. The fact that the sugars have the same T_{1m} and τ_m values for Zn- and Co-Con A implies that they are not directly bonded to the metal ion and that the conformation and sugar binding properties of the protein are not particularly sensitive to the type of ion in the transition metal binding site. In addition, no significant changes were observed in the visible spectrum of the cobalt ion in the protein upon binding of either sugar (C. F. Brewer, unpublished observation).

Binding of α- and β-MeGlc to Mn-Con A. In the presence of Mn-Con A, observed T_1 values of the ^{13}C carbons of both sugars were selectively shortened relative to their T_1 values in the presence of Zn- or Co-Con A. These results indicate that the protein bound sugars experience additional relaxation due to the presence of the paramagnetic manganese ion in the protein. The Mn^{2+} ion is an efficient source for electron–nuclear dipolar relaxation because of its relatively long electron spin–lattice relaxation time (Mildvan and Cohn, 1970). T_{1m} values for both sugars bound to Mn-Con A were calculated using eq 1b and were corrected for the contribution of τ_m to the observed T_1 values.

The paramagnetic contribution to the spin–lattice relaxation time, T_{1p}, of the ^{13}C carbons of each sugar was calculated by subtracting $(T_{1m})^{-1}$ for Zn-Con A from the $(T_{1m})^{-1}$ values

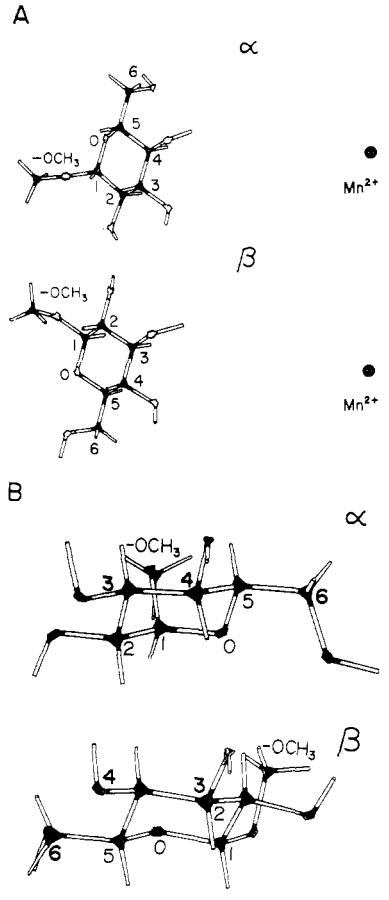

FIGURE 5: Binding orientation of α- and β-methyl D-glucopyranoside relative to the transition ion site in Con A illustrated in (A) as a side view with the Mn^{2+} ion in the plane of the paper and (B) a front view as seen from the Mn^{2+} ion. Views A and B are related by 90°. In these projections using Fieser–Dreiding models, the ring carbons and oxygen (O) are labeled. The rotamer orientations of the methoxy and hydroxyl groups for both sugars are not known.

for Mn-Con A (eq 3b). This calculation assumes that the C–H dipolar relaxation contribution in Mn-Con A is the same as that in Zn-Con A. The similarity of T_{1m} values for Co- and Zn-Con A supports this assumption.

The distance from the Mn^{2+} ion to each carbon of bound sugar was calculated using eq 4b and the T_{1p} values for the α and β anomers (Tables I and II). A value for the electron–nuclear correlation time, τ_c, in eq 4b for the Mn^{2+} ion dipolar interaction with the carbon spins of the bound sugars was determined from a study of the T_1 of solvent water protons in the presence of Mn-Con A at pH 5.60 (Koenig *et al.*, 1973). The results indicate that τ_c is dominated by τ_r at 23.4 kG. The rotational correlation time of 8×10^{-8} sec determined for the sugars bound to Zn-Con A was therefore used as the value of τ_c.

Distances calculated for the carbons of both sugars to the Mn^{2+} ion are observed to be ~10 Å (Table III), which implies that the r_{CM} vectors, *i.e.*, distance vectors from the metal ion to the sugar carbons, make approximately the same angle with the *a* semimajor axis of Con A. This justifies the use of a common isotropic rotational correlation time in calculating all of the carbon–Mn^{2+} ion distances. Relative errors of less than 5% are expected in T_{1m} values (see Theory Section). Absolute error in the distances will depend on the value of τ_r. The value for τ_r determined from the Zn-Con A data could cause an absolute error of 5–10% in the calculated r_{CM} distances. Considering the effects of rotational anisotropy of the protein and uncertainty in τ_c, absolute error in the calculated distances between the carbon atoms and Mn^{2+} ion is estimated to be <10%.

Three-Dimensional Orientation of α- and β-MeGlc Bound to Con A. A computer program was written to check the accuracy and precision of the Mn^{2+}–carbon ion distances (Table III) that were calculated from the experimental T_{1p} data and the experimentally derived value of τ_c. The computer searched for an orientation and distances of the Mn^{2+} ion with respect to the three-dimensional carbon coordinates of the sugar ring such that the ratio of the distances generated corresponded to the best fit of the ratio of the experimental T_{1p} data as related by the inverse sixth power distance relationship defined in eq 4. This provides an independent method of determining the Mn^{2+}–carbon distances for the sugars that does not require an experimental value for τ_c as was used in eq 4. The computer searched for all such orientations of the Mn^{2+} ion relative to the sugar ring while varying the Mn^{2+}–sugar distances between a mean value of 1 and 20 Å. The conformation of the bound sugars was assumed to be the same as that found for the free sugars in solution and in the crystalline state. Therefore, the coordinates of the α anomer and the *C*1 chair conformation as determined from X-ray diffraction techniques (Berman and Kim, 1968) were used for the internal bond angles of both anomers. The advantage of such a program is that it establishes whether or not the T_{1p} data are consistent with the geometrical structure of the rigid sugar ring, and if it does, at what absolute distance this agreement is greatest.

A unique orientation (Figure 5) and restricted region of Mn^{2+}–carbon distances were found for the α anomer by the computer program that were in good agreement with the distances calculated earlier from the T_{1p} data using the experimentally determined value for τ_c. The distances between the carbon atoms and Mn^{2+} ion, as generated from computer analysis, agree with the distances calculated from nmr data to within 0.2 Å for the ring carbons and to within 0.3 Å for C-6 (Table III). The agreement between the computer derived and experimental values rapidly vanishes if the distance of the sugar to the Mn^{2+} ion is varied by more than ±0.5 Å. This agreement between computer derived and experimental values strongly supports the experimental value found for τ_c and confirms the geometrical consistency of the T_{1p} data.

Two orientations were found for the β anomer. This probably results from the inability to calculate unique distances for C-3 and -5, since these resonances overlap. One orientation is similar to that shown for the α anomer except that the ring is 0.5–1.0 Å closer to the manganese ion. The computer-generated carbon–Mn^{2+} ion distances for the other orientation are listed in Table III. This orientation (Figure 5) provides a reasonable basis for explaining the difference in affinity constants of α- and β-MeGlc.

The carbon–Mn^{2+} ion distances calculated from the computer program for β-MeGlc also agree very well with the T_{1p} data and nmr calculated distances for C-1, -2, -4, and -6. Again, agreement between the computer derived and experimental values rapidly vanishes if the distance of the sugar to the Mn^{2+} ion is varied more than ±0.5 Å.

The excellent agreement between nmr calculated distances and computer generated distances is consistent with the assumption that the bound sugars remain in the *C*1 chair conformation. This result is not surprising since deformation of the pyranoside ring requires energy which would offset the binding energy of the sugars. ^{13}C nmr chemical shifts have been shown to be sensitive to pyranose steric interactions resulting from ring deformations (Perlin *et al.*, 1970). Lack of a

change in the chemical shifts of the sugar resonances in the presence of the protein is evidence for this conclusion.

The nonreducing ends of both sugars are found closest to the Mn^{2+} ion when bound to the protein. Since the ion is bound toward the interior of the protein, the nonreducing end of both sugars would appear to be directed into the Con A molecule. This conclusion is consistent with binding data that suggest that the 3-, 4-, and 6-hydroxyl groups of the α anomer are required for binding to the protein (Goldstein *et al.*, 1965b). Although both sugars bind in different orientations[2] involving the *C*1 chair conformation at a distance of 10–11 Å from the Mn^{2+} ion (Figure 5) the ring atoms of both sugars are superimposable. The 2-, 3-, and 4-hydroxyls of the β anomer bind at positions occupied by the 6-, 4-, and 3-hydroxyls of the α anomer, respectively. In essence, the pyranose ring of bound β-MeGlc is inverted and rotated relative to that of bound α-MeGlc.

In this regard, studies by Pollack and Sharon (1970) of transglycosylation reactions catalyzed by lysozyme suggest two superimposable conformations of β-D-glucose and β-D-galactose that bind to the *E* subsite of the enzyme. The two binding conformations of each sugar involved relative rotations of the sugar ring to give 1 → 4 and 1 → 2 linked oligosaccharides with glucose or galactose at the reducing end. In addition, xylose formed 1 → 4, 1 → 3, and 1 → 2 linkages in transglycosylation reactions, suggesting three different binding orientations of this sugar at the *E* subsite.

Interactions of α- and β-MeGlc and Their Derivatives with Con A. The binding orientation of β-MeGlc apparently reflects an unfavorable steric interaction between the protein and the equatorial methoxy group of the sugar when it binds in the orientation found for α-MeGlc (Poretz and Goldstein, 1970). Consequently, β-MeGlc binds in such a way that its methoxy group is rotated away from this unfavorable steric interaction to a position over the ring oxygen of bound α-MeGlc. In this orientation (Figure 5), the 6-hydroxylmethyl group of β-MeGlc binds in the position occupied by the 2-hydroxyl of α-MeGlc. Although the hydroxymethyl group is larger than the hydroxyl group, there appears to be sufficient steric tolerance for it in this region of the protein based on a study of the binding of a series of 2-substituted derivatives of α-MeGlc to Con A (Poretz and Goldstein, 1970).

The 24-fold greater affinity of α-MeGlc for Con A than β-MeGlc can be related to the respective binding orientations and interactions of these sugars with the protein. Consideration of relative affinity constants of certain modified derivatives of both sugars allows assessment of these different interactions. In the following analysis we assume that (a) replacement of a hydroxyl group in the sugars by a nonhydrogen bonding substituent results in a loss of hydrogen bonding with Con A at that position and (b) the contribution to binding remains unchanged for the unmodified portion of the sugar molecule.

The relative binding constants of derivatives of the two anomers can be considered in the following manner. The oxygen of the methoxy group of α-MeGlc appears to enhance binding by a factor of 5 relative to the corresponding 1-deoxy analog, 1,5-anhydro-D-glucitol (Poretz and Goldstein, 1970). However, the oxygen of the methoxy group of β-MeGlc is rotated out of this region which most likely results in the loss of this interaction. The 2-hydroxyl of α-MeGlc destabilizes binding by a factor of approximately 2 relative to the 2-deoxy analog, α-methyl 2-deoxy-D-glucopyranoside (Poretz and Goldstein, 1970). However, the axial 2-hydroxyl of α-methyl D-mannopyranoside enhances binding by a factor of 3 relative to the 2-deoxy analog (Poretz and Goldstein, 1970), which suggests that a protein binding site for the sugar exists at this position. Furthermore, the 6-hydroxymethyl group of β-MeGlc, which binds at the 2-hydroxyl position of α-MeGlc, increases binding by a factor of 11 relative to the analog lacking the 6-hydroxymethyl group (β-methyl D-xylopyranoside) (So and Goldstein, 1968). Thus, it appears that the 6-hydroxymethyl group of β-MeGlc binds 22 times better to Con A than when the 2-hydroxyl of α-MeGlc is positioned at this site in the protein. Since the 4- and 3-hydroxyls of β-MeGlc bind at positions respectively occupied by the 3- and 4-hydroxyls of α-MeGlc, their binding energies for both anomers should be the same.

The difference in binding energy between the 2-hydroxyl of β-MeGlc and the 6-hydroxylmethyl group of α-MeGlc, which are both proposed to occupy the same binding site, can also be determined. The binding of α-methyl 6-deoxy-6-iodo-D-glucopyranoside (6-iodo-α-MeGlc) is nearly 200 times less than α-MeGlc (So and Goldstein, 1967), and substitution of the entire 6-hydroxymethyl group of α-MeGlc by a hydrogen (α-methyl D-xylopyranoside) results in a derivative which binds with essentially the same affinity as 6-iodo-α-MeGlc (So and Goldstein, 1968). Thus, substitution of an iodine atom for the 6-hydroxyl of α-MeGlc eliminates binding at this position. Substitution of an iodine for the 2-hydroxyl of β-MeGlc to give β-methyl 2-deoxy-2-iodo-D-glucopyranoside (2-iodo-β-MeGlc) decreases binding by only a factor of 2 (So and Goldstein, 1967). Assuming that substitution of the iodine atom for the 2-hydroxyl of β-MeGlc also results in a complete loss of binding at this position, the 2-hydroxyl of β-MeGlc binds 100 times less than that of the 6-hydroxymethyl group of α-MeGlc at this site in the protein.

Thus, β-MeGlc binds with the following interactions that differ from those of α-MeGlc: a 100-fold loss in binding by the 2-hydroxyl at the site occupied by the 6-hydroxymethyl group of α-MeGlc, a fivefold loss in binding by the oxygen atom of the methoxy group, and a 22-fold enhancement in binding by the 6-hydroxymethyl group at the site occupied by the 2-hydroxyl of α-MeGlc. Summated, the relative binding constant for β-MeGlc is estimated to be 23 times less than that of α-MeGlc, compared to the experimentally determined value of 24. The difference in affinity constants between α- and β-MeGlc is assumed to be primarily due to weak binding of the 2-hydroxyl of β-MeGlc at the position in the protein which binds the 6-hydroxymethyl group of α-MeGlc.

The relative affinity constants of derivatives of both sugars can also be explained by the different binding interactions of the two anomers. For example, removal of the 6-hydroxymethyl group of both anomers to give the corresponding α- and β-methyl D-xylopyranosides results in a 270-fold loss in binding for the α anomer, but only an 11-fold loss in binding for the β anomer (So and Goldstein, 1969). This is consistent with the postulate that the 6-hydroxymethyl group of both anomers binds to different sites in the protein. In addition, such sugars as α-methyl L-sorbopyranoside and β-D-fructopyranoside that bind well to Con A can be rotated into orientation such that their structures are nearly superimposable on

[2] The possibility that the manganese ion moves to different positions in the protein relative to the carbohydrate binding site, depending upon which of the two sugars is bound, can be excluded from epr measurements of Mn-Con A which showed no change in the spectrum upon the addition of either sugar. In addition, the visible spectrum of Co-Con A was not disturbed when either sugar was bound to the protein (C. F. Brewer, unpublished results).

the glucopyranoside ring, as suggested by So and Goldstein (1969).

The binding of α- and β-MeGlc to Con A also suggests a source of specificity for oligo- and polysaccharide binding to proteins that, like Con A, bind primarily to the nonreducing terminal sugar. The different binding orientations of the two anomers suggest different spatial orientations of polysaccharide units connected to either of these sugars when they bind to the protein. This difference may either contribute or diminish the binding energy of the polysaccharide. Indeed, polysaccharides with α-linked terminal mannopyranoside or glucopyranoside residues bind well to Con A, but corresponding β-linked polysaccharides show no ability to bind to Con A (Goldstein *et al.*, 1965a).

Comparison of Solution and Crystalline Con A–Saccharide Complexes. The mean value of 10 Å separating the Mn^{2+} ion from bound α- or β-MeGlc in solution contrasts with values for this distance derived from X-ray diffraction studies of crystalline Con A–saccharide complexes. The possibility that the Mn^{2+} ion which influences the T_1 rates of the bound sugars is located at some secondary binding site on the protein's surface is unlikely for several reasons that have been discussed elsewhere (Brewer *et al.*, 1973). In the crystalline complex of *myo*-inositol and Con A the cyclohexane ring of the sugar is 23 Å from the Mn^{2+} ion (Hardman and Ainsworth, 1972). However, it should be noted that *myo*-inositol does not inhibit hemagglutination by Con A or the precipitation of dextrans by Con A (Edelman *et al.*, 1972). Since binding specificity of the protein in the crystalline state is not known, the precise relationship of the binding of *myo*-inositol to crystalline Con A to the binding of sugars such as α- and β-MeGlc to Con A in solution remains to be established.

Edelman *et al.* (1972) report a value of 20 Å for the distance between the sugar binding site and the transition metal binding site based on X-ray crystallographic studies of the β-(*o*-iodophenyl) D-glucopyranoside–Con A crystalline complex. The iodine atom of β-(*o*-iodophenyl) D-glucopyranoside is located in the major cleft of the protein at nearly the same site as the cyclohexanol ring of *myo*-inositol. The glucopyranoside ring was not directly observed but its position was inferred to be in the cleft above the iodine atom, with its nonreducing end facing the surface of the protein and directed away from the Mn^{2+} ion. If binding occurs in the major cleft, our data suggest that both α- and β-MeGlc bind near the bottom of this cleft at a mean distance of 10–11 Å from the Mn^{2+} ion with their nonreducing ends facing this ion. However, at present the data do not rule out the possibility that the carbohydrate binding site may be located elsewhere in the protein.

Circular dichroism measurements indicate that Con A undergoes some conformational change in solution when α-methyl D-mannopyranoside (Pflumm *et al.*, 1971) or α-MeGlc (C. F. Brewer and I. Listowsky, unpublished data) are bound. The cracking and dissolving of crystals of Con A upon exposure to α-methyl D-mannopyranoside (K. D. Hardman, personal communication) may be caused by this conformational change in the complex. Certain proteins have been suggested to exist in a different conformation in solution than in the crystalline form. For example, crystalline carboxypeptidase A possesses only 0.3% of the specific activity of the enzyme in solution (Quiocho and Richards, 1966), and Johansen and Vallee (1971) have presented additional evidence for a difference in conformation between the crystalline and solution structure of carboxypeptidase A.

Calculations of internuclear distances have previously been made from proton, fluorine, and phosphorus nmr measurements of small molecules complexed with paramagnetic ion (*cf.* Shulman *et al.*, 1965) as well as small molecules bound to proteins containing paramagnetic ions (Mildvan and Cohn, 1970; Bennick *et al.*, 1971; and Butchard *et al.*, 1972). ^{13}C nmr, as demonstrated in our study and others (Fung *et al.*, 1973), extends this approach to determine the three-dimensional orientation of small molecules bound to proteins in solution. These techniques can be applied to a variety of small molecules, including drugs, that bind to macromolecules containing a binding site for paramagnetic ions. Such ^{13}C nmr measurements should define the binding site and provide information on the solution structure of the complex. When X-ray diffraction data are available for the small molecule–macromolecule complex, a comparison of the structure of the complex in solution and in the crystalline form can also be made.

Binding of α- and β-MeGlc by Con A may serve as a model for other proteins, such as enzymes and antibodies, that bind saccharides. Structure–activity correlations for compounds or drugs that bind to macromolecules are frequently difficult to interpret, since a change in the orientation of a single substituent, as suggested in the case of α- and β-MeGlc, can drastically alter the orientation and interactions of the small molecule with the protein. The different orientations determined for the binding of α- and β-MeGlc to Con A illustrate the value of nmr techniques for correlating structure–activity relationships among small molecules that bind to macromolecules.

Acknowledgments

The authors wish to thank Dr. Allen Tonelli of Bell Laboratories, Murray Hill, N. J., for his assistance in writing the computer programs used in this study and Dr. Gerald Edelman for allowing us to inspect his Kendrew model of Con A. The authors also wish to acknowledge the many helpful conversations with Dr. Karl Hardman and Dr. Irwin Goldstein. This is Publication No. 306 from the Joan and Lester Avnet Institute of Molecular Biology.

Addendum

We have synthesized β-(*o*-iodophenyl) D-glucopyranoside, uniformly labeled with 14% ^{13}C in the sugar moiety, and used the ^{13}C nmr techniques described in this paper to demonstrate that a fraction of this aryl glycoside binds to Mn-Con A with the nonreducing end of the sugar moiety directed toward the Mn^{2+} ion at a mean distance of 10 Å. Additional experiments indicate that some of the remaining β-iodophenyl glucopyranoside binds at a second site in the protein which may relate to the site discussed by Edelman *et al.* (1972) for the crystalline complex. In addition, C. F. Brewer and K. D. Hardman (unpublished data) have demonstrated that β-(*o*-iodophenyl) D-galactopyranoside, an analog of β-(*o*-iodophenyl) D-glucopyranoside that does not inhibit hemagglutination or dextran precipitation by Con A, forms a crystalline complex with Con A in which the iodine atom of β-(*o*-iodophenyl) D-galactopyranoside occupies the same site in the protein as that of β-(*o*-iodophenyl) D-glucopyranoside. Our results suggest that the iodophenyl ring rather than the sacharide moiety is principally responsible for the binding of β-(*o*-iodophenyl) D-glucopyranoside and β-(*o*-iodophenyl) D-galactopyranoside to crystalline Con A.

References

Abragam, A. (1961), Principles of Nuclear Magnetism, New York, N. Y., Oxford University Press, Chapter 8.

Austin, P. W., Hardy, F. E., Buchanan, J. G., and Baddiley, J. (1963), *J. Chem. Soc. 5*, 5350.

Bartlett, G. M., and Sheppard, G. (1969), *J. Label. Compounds 5*, 275.

Bennick, A., Campbell, I. D., Dwek, R. A., Price, N. C., Radda, G. K., and Salomon, A. G. (1971), *Nature (London) 234*, 140.

Berman, H. M., and Kim, S. H. (1968), *Acta. Crystallogr. 24*, 897.

Bloembergen, N., and Morgan, L. O. (1961), *J. Chem. Phys. 34*, 307.

Brewer, C. F., Sternlicht, H., Marcus, D. M., and Grollman, A. P. (1972), *in* Lysozyme, Osserman, E., Beychok, S., and Canfield, R., Ed., New York, N. Y., Academic Press (in press).

Brewer, C. F., Sternlicht, H., Marcus, D. M., and Grollman, A. P. (1973), *Proc. Nat. Acad. Sci. U. S. 70*, 1007.

Burger, M. M., and Noonan, K. D. (1970), *Nature (London) 228*, 512.

Butchard, C. G., Dwek, R. A., Kent, P. W., Williams, R. J. P., and Xavier, A. U. (1972), *Eur. J. Biochem. 27*, 548.

Conchie, J., Levy, G. A., and Marsh, C. A. (1957), *Advan. Carbohyd. Chem. 12*, 157.

Dahlquist, F. W., and Raftery, M. A. (1969), *Biochemistry 8*, 713.

Doddrell, D., Glushko, V., and Allerhand, A. (1972), *J. Chem. Phys. 56*, 3683.

Dorman, D. E., and Roberts, J. D. (1970), *J. Amer. Chem. Soc. 92*, 1355.

Edelman, G. M., Cunningham, B. A., Reeke, G. N., Jr., Becker, J. W., Waxdal, M. J., and Wang, J. L. (1972), *Proc. Nat. Acad. Sci. U. S. 69*, 2580.

Eisinger, J., Shulman, R. G., and Szymanski, B. M. (1962), *J. Chem. Phys. 36*, 1721.

Fung, C. H., Mildvan, A. S., Allerhand, A., Komoroski, R., and Scrutton, M. C. (1973), *Biochemistry 12*, 620.

Goldstein, I. J., Hollerman, C. E., and Merrick, J. M. (1965a), *Biochim. Biophys. Acta 97*, 68.

Goldstein, I. J., Hollerman, C. E., and Smith, E. E. (1965b), *Biochemistry 4*, 876.

Hardman, K. D., and Ainsworth, C. F. (1972), *Biochemistry 11*, 4910.

Inbar, M., and Sachs, L. (1969), *Proc. Nat. Acad. Sci. U. S. 63*, 1418.

Johansen, J. T., and Vallee, B. L. (1971), *Proc. Nat. Acad. Sci. U. S. 68*, 2532.

Kalb, A. J., and Levitzki, A. (1968), *Biochem. J. 109*, 669.

Kalb, A. J., and Lustig, A. (1968), *Biochim. Biophys. Acta 168*, 366.

Koenig, S., Brown, R., and Brewer, C. F. (1973), *Proc. Nat. Acad. Sci. U. S. 70*, 475.

Marcus, D. M., and Grollman, A. P. (1966), *J. Immunol. 97*, 867.

McKenzie, G. H., Sawyer, W. H., and Nichol, L. W. (1972), *Biochim. Biophys. Acta 263*, 283.

Mildvan, A., and Cohn, M. (1970), *Advan. Enzymol. 33*, 1.

Millett, F., and Raftery, M. A. (1972), *Biochemistry 11*, 1639.

Perlin, A. S., Casu, B., and Koch, H. J. (1970), *Can. J. Chem. 48*, 2596.

Perrin, F. (1936), *J. Phys. Radium 7*, 1.

Pflumm, M. N., Wang J. L., and Edelman, G. M. (1971), *J. Biol. Chem. 246*, 4369.

Pollock, J. J., and Sharon, N. (1970), *Biochemistry 9*, 3913.

Poretz, R. D., and Goldstein, I. J. (1970), *Biochemistry 9*, 2890.

Quiocho, F. A., and Richards, F. M. (1966), *Biochemistry 5*, 4062.

Reed, G. H., and Cohn, M. (1970), *J. Biol. Chem. 245*, 662.

Rubenstein, M., Baram, A., and Luz, Z. (1971), *Mol. Phys. 20*, 67.

Sharon, N., and Lis, H. (1972), *Science 177*, 946.

Shoham, J., Inbar, M., and Sachs, L. (1970), *Nature (London) 227*, 1244.

Shulman, R. G., Sternlicht, H., and Wyluda, B. J. (1965), *J. Chem. Phys. 43*, 3116.

So, L. L., and Goldstein, I. J. (1967), *J. Immunol. 99*, 158.

So, L. L., and Goldstein, I. J. (1968), *Biochim. Biophys. Acta 165*, 398.

So, L. L., and Goldstein, I. J. (1969), *Carbohyd. Res. 10*, 231.

Sumner, J. B., and Howell, S. F. (1936), *J. Bacteriol. 32*, 227.

Swift, T. J., and Connick, R. E. (1962), *J. Chem. Phys. 37*, 307.

Vold, R. L., Waugh, J. J., Klein, M. P., and Phelps, D. E. (1968), *J. Chem. Phys. 48*, 3831.

Weinzierl, J., and Kalb, A. J. (1971), *FEBS (Fed. Eur. Biochem. Soc.) Lett. 18*, 268.

Woessner, D. E. (1962), *J. Chem. Phys. 37*, 647.