An Examination of the Topography of the Saccharide Binding Sites of Concanavalin A and of the Forces Involved in Complexation*

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ABSTRACT: The topography of the saccharide binding region of concanavalin A and the forces involved in the stabilization of the complex of sugar with this hemagglutinin was systematically explored utilizing the quantitative hapten inhibition technique. A variety of deoxy, *O*-alkyl, halogeno, thio, and acetamido derivatives of D-glucose and D-mannose were employed in these studies. Data were obtained which indicated that: (a) this protein possesses specific binding loci capable of interacting with the *oxygen* atoms of the C-1, C-2, and C-3 hydroxyl groups as well as the hydroxyl moiety of the C-4 portion of α-D-mannopyranosyl residues; (b) the protein is in close proximity to the β-glycosidic oxygen atom and the C-2 hydroxyl group of bound β-D-glucopyranosyl residues, but does not bind to the carbohydrate at these points; (c) the saccharide probably binds to the protein when in a C-1 chain conformation; (d) calculation of the contribution of each hydroxyl group to the total change in free energy of binding of methyl α-D-mannopyranoside to cancanavalin A was performed. Summation of the $\Delta(\Delta F^{\circ\prime})$ values led to a value (−9.9 kcal/mole) which was −4.5 kcal/mole higher than that obtained by equilibrium dialysis (5.4 kcal/mole).

Studies employing alkyl β-D-glucopyranosides as inhibitors suggest that the region of cancanavalin A in juxtaposition to the aglycone of the bound glycoside may accommodate branching at the β but not at the α-carbon atom of the alkyl aglycone.

Initial studies (Goldstein *et al.*, 1965) designed to describe the saccharide binding region of concanavalin A demonstrated that the 2-deoxy-1,5-anhydro-D-*arabino*-hexitol molecule apparently contains the minimum configurational features required for interaction with this protein, namely, unmodified hydroxyl groups at the C-3, C-4, and C-6 positions of the indicated configuration. Furthermore, the combining sites of concanavalin A were shown to have a high degree of specificity for the α configuration at the anomeric carbon atom of the D-pyranose ring system and that the D-*manno* configuration was bound more strongly than the D-*gluco* configuration.

Recently So and Goldstein (1969) reported that 1,4-anhydro-D-arabinitol, which contains the tetrahydrofuran ring system, will also bind to concanavalin A, the disposition of the hydroxyl groups at C-2, C-3, and C-5 bearing a formal configurational relationship to the corresponding hydroxyl groups at positions C-3, C-4, and C-6 of D-glucopyranose.

In order to calculate the contribution of each portion of the carbohydrate molecule to the total binding energy of the concanavalin A–saccharide complex and to ascertain precisely which atoms of the sugar molecule are involved directly in binding to the concanavalin A molecule, we have examined the inhibiting capacity of a large number of derivatives of D-glucose and D-mannose modified at the C-1, C-2, C-3, and C-4 positions. These derivatives include various α- and β-glycosides of deoxy sugars lacking the hydroxyl groups under question, sugars with halogen atoms or acetamido groups in place of hydroxyl groups, and sugars possessing an alkyl residue in place of the hydrogen atom of the appropriate hydroxyl function (*O*-alkyl sugars).

We have also examined the binding to concanavalin A of a series of alkyl β-D-glucopyranosides in order to explore the topography of that portion of the concanavalin A molecule which is in juxtaposition to the aglycone of the bound saccharide.

Materials and Methods

Ethyl 1-Thio-β-D-glucopyranoside. Ethyl 1-thio-β-D-glucopyranoside was prepared by means of its peracetate by the method of Lemieux (1951). The hygroscopic product had mp 97–100° and $[\alpha]_D^{23}$ −63.1° (*c* 1.5, H_2O) (lit. (Lemieux, 1951) mp 99–100°, $[\alpha]_D$ −55.14°).

1-Phenyl-1,5-anhydro-α-D-glucitol and 1-Phenyl-1,5-anhydro-β-D-glucitol. The anomeric 1-phenyl-1,5-anhydro-D-glucitols were synthesized as described by Bonner (*cf.* 1951; Hurd and Bonner, 1945; Bonner and Craig, 1950). The α anomer had mp 185–187° (lit. (Bonner and Craig, 1950) mp 187°) and the β anomer was obtained as a clear colorless syrup, $[\alpha]_D^{23}$ +16.8° (*c* 1.2, H_2O) (lit. (Bonner, 1951) $[\alpha]_D$ +18.6°).

Racemic 1,2-O-(1-Methoxyethylidene)-β-D-mannopyranose. The appropriate orthoacetate of D-mannose was deacetylated by the general method of Zemplen and Pacsu (1929) to yield 1,2-*O*-(1-methoxyethylidene)-β-D-mannopyranose. Additional purification of the product was accomplished by

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preparative column chromatography (2 × 70 cm) utilizing silicic acid (Mallinckrodt Chemical Works, St. Louis, Mo.) as the stationary support and elution with an azeotrope of butanone–water. This procedure yielded a clear, colorless syrup $[\alpha]_D^{23}$ −9.1° (*c* 2.3, 50% aqueous ethanol (lit. (Bott *et al.*, 1930) $[\alpha]_D$ −6.0°).

2-O-Methol-O-mannose. 2-*O*-Methyl-D-mannose was prepared by a procedure to be described elsewhere (Poretz, 1968). It had mp 138–139.5°, $[\alpha]_D^{23}$ +3.3° (*c* 1.0, H_2O) (lit. (Pacsu and Trister, 1941) mp 136–137°, $[\alpha]_D$ +7.0–4.5°).

Alkyl and Aralkyl β-D-glucopyranosides. The alkyl and aralkyl 2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosides were prepared by variations of the general procedure of Koenigs and Knorr (1901a,b; *cf.* Bates and Associates, 1942). The corresponding deacetylated products were obtained by the method of Zemplen and Pacsu (1929). Of the glycosides discussed in this paper, all have been reported previously except for 2′,2′,2′-trifluoroethyl 2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranoside (mp 137–8° recrystallized from ethanol; $[\alpha]_D^{23}$ −22.1°, *c* 2.1, $CHCl_3$; *Anal.* Calcd for $C_{16}H_{21}O_{10}F_3$: C, 44.65; H, 4.92; F, 13.25. Found: C, 44.68; H, 4.93; F, 13.34.) and its deacetylated product (mp 146–148°, recrystallized from ethyl acetate; $[\alpha]_D^{23}$ −30.6°, *c* 1.4, H_2O; *Anal.* Calcd for $C_8H_{13}O_6F_3$: C, 36.65; H, 5.00; F, 21.74. Found: C, 36.50; H, 4.85; F, 21.52.), and cyclopentyl β-D-glucopyranoside (mp 98–100°, recrystallized from ethyl acetate, $[\alpha]_D^{23}$ −50.3°, *c* 1.1, H_2O).

Phenyl β-Cellobioside. The method of Montgomery *et al.* (1943) was followed for the preparation of phenyl β-cellobioside.

1,5-Anhydro-D-glucitol, 1,5-anhydro-D-mannitol, phenyl 3-deoxy-β-D-*ribo*-hexopyranoside, and methyl 4,6-*O*-benzylidene-3-deoxy-α-D-*arabino*-hexopyranoside were the gift of Dr. N. K. Richtmyer. Methyl 4-deoxy-α-D-*xylo*-hexopyranoside was supplied by Dr. E. J. Hedgley, β-D-mannose 1,2-(methyl orthoacetate) triacetate was a gift of Dr. R. Montgomery. Methyl 2-*O*-isopropyl- (2-*O*-methyl- and 2-*O*-ethyl-) α-D-glucopyranoside were the gift of Dr. B. Lindberg; 3-deoxy-3-fluoro-D-glucose was the gift of Dr. A. B. Foster; methyl 2-chloro-2-deoxy-β-D-glucopyranoside, methyl 2-deoxy-2-iodo-β-D-glucopyranoside, and methyl 2-deoxy-α-D-*arabino*-hexopyranoside were obtained from Dr. R. U. Lemieux. The aromatic thioglycosides were obtained from Dr. M. Jermyn. Methyl 2-*O*-(2-cyanoethyl)-α-D-glucopyranoside was supplied by Dr. P. J. Garegg. Isobutyl and neopentyl β-D-glucopyranosides were the gift of Professor T. Timell, and cyclohexyl β-D-glucopyranoside was kindly provided by Dr. R. Iyer. The remaining saccharides were obtained commercially.

All compounds migrated as a single component on thin-layer chromatography using silica gel G (Brinkman Instruments, Westbury, N. Y.) and irrigation with either azeotropic butanone–water, ethyl acetate–isopropyl alcohol–water (65:23:12, v/v), or 1-propanol–ethyl acetate–water (3:2:1, v/v). Components were visualized by charring with 50% ethanolic sulfuric acid and heating at 110° for 10 min.

Inhibition Assay. The quantitative turbidimetric procedure using levan NRRL B-512 as the precipitating polysaccharide was employed for inhibition analysis (Poretz and Goldstein, 1967, 1968).

Concanavalin A. Purified concanavalin A was prepared by the general procedure of Agrawal and Goldstein (1965, 1967), except that the ammonium sulfate precipitation step was omitted. The final product was 97–98% precipitable by levan B-512 and displayed one band on cellulose acetate (Seprophore, Gelman Ind., Ann Arbor, Mich.), electrophoresis at pH 5.0, 250 V for 1.5 hr (anodic mobility 1.0 mm).

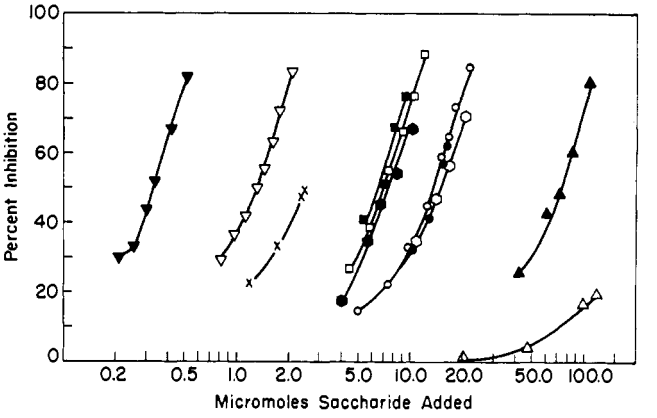

FIGURE 1: Inhibition of concanavalin A–levan interaction by C-1 derivatives of D-mannose and D-glucose. Methyl α-D-mannopyranoside (▼), methyl α-D-glucopyranoside (▽), 1,5-anhydro-D-mannitol (×), *p*-nitrophenyl 1-thio-β-D-glucopyranoside (■), phenyl β-D-glucopyranoside (□), 1,5-anhydro-D-glucitol (◆), phenyl-1-thio-β-D-glucopyranoside (○), methyl β-D-mannopyranoside (●), *p*-methylphenyl β-D-glucopyranoside (○), ethyl 1-thio-β-D-glucopyranoside (▲), and β-D-glucoypranoside phenyl sulfone (△). Concanavalin A (320 μg) and levan B-512 (200 μg).

Results

Figure 1 represents a series of curves generated when the percentage inhibition of the concanavalin A–levan interaction is plotted with respect to the common logarithm of the amount of inhibitor added.

Table I compares the amount of material required for 50% inhibition produced by a number of the C-1 derivatives displayed in Figure 1 in addition to several further compounds. Methyl α-D-mannopyranoside was approximately 7.5 times more effective as an inhibitor than the analogous compound lacking the C-1 methoxyl group, namely, 1,5-anhydro-D-mannitol. However, the presence of a β-*O*-methyl function, as in methyl β-D-mannopyranoside caused a decrease of 5.6 times in the inhibiting potency of this compound. A similar relationship exists in the D-glucoside series.

A tabulation of the inhibiting potencies of a variety of 1-thio-β-D-glucopyranosides and analogous *O*-β-D-glucopyranosides is also shown in Table I. It will be noted that substitution of the oxygen atom at the C-1 position of ethyl β-D-glucopyranoside by a sulfur atom (ethyl 1-thio-β-D-glucopyranoside) produced a twofold decrease in the inhibiting potency of this glycoside. In similar fashion, phenyl 1-thio-β-D-glucopyranoside required 13.5 μmoles for 50% inhibition as compared to phenyl β-D-glucopyranosides which required only 7.0 μmoles for the same extent of inhibition.

Comparison of a series of phenyl glycosides and their thio analogs indicates the *O*-glycosides to be better inhibitors. Nevertheless *p*-nitrophenyl 1-thio-D-β-glucopyranoside has approximately the same inhibiting potency (7.1 μmoles for 50% inhibition) as *p*-nitrophenyl β-D-glucopyranoside (6.5 μmoles for 50% inhibition).

TABLE I: Inhibiting Power of Various C-1 Derivatives of D-Glucose and D-Mannose.

Compound	μmoles for 50% Inhibn
1,5-Anhydro-D-mannitol	2.5
Methyl α-D-mannopyranoside	0.34
Methyl β-D-mannopyranoside	14.0
1,5-Anhydro-D-glucitol	7.2
Methyl α-D-glucopyranoside	1.3
Methyl β-D-glucopyranoside	37
Ethyl β-D-glucopyranoside	37
Ethyl 1-thio-α-D-glucopyranoside	73
Phenyl β-D-glucopyranoside	7.0
Phenyl 1-thio-β-D-glucopyranoside	13.5
p-Methylphenyl β-D-glucopyranoside	6.5
p-Methylphenyl 1-thio-β-D-glucopyranoside	15.0
p-Nitrophenyl β-D-glucopyranoside	6.5
p-Nitrophenyl 1-thio-β-D-glucopyranoside	7.1
β-D-Glucopyranoside phenyl sulfone	115 (20%)[a]
Phenyl 1,5-anhydro-β-D-glucitol	1.3
Phenyl 1,5-anhydro-β-D-glucitol	5.5

[a] Numbers in parentheses refer to percentage inhibition given by micromoles of saccharide noted.

Tables II and III indicate, as reported previously, that D-mannose derivatives are more potent inhibitors of the concanavalin A–polysaccharide interaction than the analogous D-glucose compounds (Goldstein *et al.*, 1965; Smith and Goldstein, 1967; Poretz and Goldstein, 1967, 1968; So and Goldstein, 1967a). If we consider methyl 2-deoxy-α-D-*arabino*-hexopyranoside as parent compound, the introduction of a C-2 hydroxyl group of the D-*manno* configuration (methyl α-D-mannopyranoside) produced a threefold increase in inhibiting power. However, introduction of a C-2 hydroxyl group of the D-*gluco* configuration (methyl α-D-glucopyranoside) resulted in a decrease in the inhibiting potency of the saccharide.

TABLE II: Inhibiting Power of Some C-2 Derivatives of D-Glucose.

Compound	μmoles for 50% Inhibn
Methyl 2-deoxy-α-D-*arabino*-hexopyranoside	0.95
Methyl α-D-mannopyranoside	0.34
Methyl α-D-glucopyranoside	1.3
Methyl 2-*O*-methyl-α-D-glucopyranoside	1.1
Methyl 2-*O*-ethyl-α-D-glucopyranoside	1.3
Methyl 2-*O*-isopropyl-α-D-glucopyranoside	2.5
Methyl 2-*O*-(2-cyanoethyl)-α-D-glucopyranoside	2.3
Methyl 2-acetamido-2-deoxy-α-D-glucopyranoside	2.8
Methyl 2-chloro-2-deoxy-α-D-glucopryranoside	10
Phenyl α-D-glucopyranoside	0.56
Phenyl 2-acetamido-2-deoxy-α-D-glucopyranoside	0.95
Methyl β-D-glucopyranoside	37
Methyl 2-deoxy-2-iodo-β-D-glucopyranoside	68

TABLE III: Inhibiting Power of C-2 Derivatives of D-Mannose.

Compound	μmoles for 50% Inhibn
p-Nitrophenyl α-D-mannopyranoside	0.17
p-Nitrophenyl 2-*O*-methyl-α-D-mannopyranoside	0.19
D-Mannose	2.0
2-*O*-Methyl-D-mannose	1.9
2-Acetamido-2-deoxy-D-mannose	9.5 (4%)[a]
1,2-*O*-(1-Methoxyethylidene)-β-D-mannopyranoside	2.4

[a] Numbers in parentheses refer to percentage inhibition given by micromoles of saccharide noted.

As shown in Table II, a series of C-2 derivatives of D-glucose and methyl α-D-glucopyranoside was studied in order to explore the stereochemistry of the protein combining site complementary to the C-2 position. Addition of a 2-*O*-methyl or a 2-*O*-ethyl group has little effect on the inhibiting potency of methyl α-D-glucopyranoside whereas the more bulky 2-*O*-isopropyl group caused a twofold decrease in the potency of this derivative. Similarly, both methyl 2-*O*-cyanoethyl α-D-glucopyranoside and methyl 2-acetamido-2-deoxy-α-D-glucopyranoside were only one-half as potent inhibitors as methyl α-D-glucopyranoside. Methyl 2-chloro-2-deoxy-α-D-glucopyranoside is only about one-tenth as potent as methyl 2-deoxy-α-D-*arabino*-hexopyranoside. Furthermore, it appears that the analogous phenyl α-D-glucopyranoside closely parallel the methyl glucopyranoside series. This effect appears to be related to the substituent at the C-2 position (in the D-*gluco* configuration) of methyl 2-deoxy-α-D-*arabino*-hexopyranoside in the following manner: hydro > hydroxy = methoxy = ethoxy > isopropoxy = 2-cyanoethyl > acetamido ≫ chloro.

Of the derivatives of D-mannose in Table III, both *p*-nitrophenyl α-D-mannopyranoside and its 2-*O*-methyl analog are equally potent as inhibitors of the concanavalin A–levan interaction. Similar result were obtained with D-mannose and its 2-*O*-methyl derivative, which required 2.0 and 1.9 μmoles, respectively, for 50% inhibition. Furthermore, replacement of the C-2 hydroxyl group of D-mannose by an acetamido group (2-acetamido-2-deoxy-D-mannose) resulted in a very poor inhibitor (9.5 μmoles gave only 4% inhibition).

The inhibiting potencies of C-3 and C-4 derivative of D-mannose and D-glucose are shown in Table IV. The almost absolute requirement for the C-3 hydroxyl group is demonstrated by the 70-fold difference in inhibiting potency of methyl α-D-mannopyranoside and its 3-deoxy derivative (methyl 3-deoxy-α-D-*arabino*-hexopyranoside). Similarly, phenyl 3-deoxy-β-D-*ribo*-hexopyranoside which produced negligible inhibition up to 123 moles may be compared

with phenyl β-D-glucopyranoside which required 7.0 μmoles for 50% inhibition. On the other hand, a comparison of methyl α-D-glucopyranoside and methyl 3-*O*-methyl-α-D-glucopyranoside revealed that only a 26-fold decrease in inhibiting power occurred upon substitution of the hydrogen atom of the C-3 hydroxyl group by a methyl group.

3-Deoxy-3-fluoro-D-glucose is only 1.5 times less active as an inhibitor than D-glucose, these sugars requiring 16.6 and 11.3 μmoles, respectively, for 50% inhibition. Methyl 4-deoxy-α-D-*xylo*-hexopyranoside (Table IV) is at least 265 times less potent than methyl α-D-glucopyranoside.

With regard to the relationship of the aglycone to the binding of glycosides to concanavalin A it is apparent from Table V that the β-D-glucopyranosides of the primary alcohols required 35–37 μmoles for 50% inhibition. (A notable exception is 2′,2′,2′-trifluoroethyl β-D-glucopyranoside which required 59 μmoles for 50% inhibition.) However, isopropyl β-D-glucopyranoside required 100 μmoles for 50% inhibition and the *t*-butyl glycoside required 165 μmoles.

Cyclohexyl β-D-glucopyranoside, which possesses features of the isopropyl group in which the methylene groups have limited freedom of rotation, required 65 μmoles for 50% inhibition and is 1.5 times more effective as an inhibitor than isopropyl β-D-glucopyranoside. Cyclopentyl β-D-glucopyranoside required only 46 μmoles for 50% inhibition. It should be noted that phenyl β-D-glucopyranoside has an inhibiting power approximately five times greater than methyl β-D-glucopyranoside and nearly ten times that of cyclohexyl β-D-glucopyranoside.

Some β-glucobioses are also included in Table V for comparison with the β-D-glucopyranosides containing simple aglycones. Gentiobiose, a β-(1→6)-linked glucobiose (containing a glycosidic bond to a primary alcoholic function) is a more potent inhibitor than cellobiose, a β-(1→4)-linked disaccharide which possesses a glycosidic bond which involves a secondary alcoholic grouping. Similarly, amygdalin, the gentiobioside of mandelonitrile inhibited concanavalin A–levan interaction more readily than phenyl β-cellobioside.

TABLE IV: Inhibiting Power of C-3 and C-4 Derivatives of D-Mannose and D-Glucose.

Compound	μmoles for 50% Inhibn
Methyl α-D-mannopyranoside	0.34
Methyl 3-deoxy-α-D-*arabino*-hexopyranoside	24
Phenyl β-D-glucopyranoside	7.0
Phenyl 3-deoxy-β-D-*ribo*-hexopyranoside	123 (2%)[a]
Methyl α-D-glucopyranoside	1.3
Methyl 3-*O*-methyl-α-D-glucopyranoside	34
Methyl 4-deoxy-α-D-*xylo*-hexopyranoside	99 (0%)
D-Glucose	11.3
3-Deoxy-3-fluoro-D-glucose	16.6

[a] Numbers in parentheses refer to percentage inhibition given by micromoles of saccharide noted.

Discussion

A primary goal in this study has been to describe in detail the noncovalent forces which are involved in the binding of carbohydrates to concanavalin A, the phytohemagglutinin of the jack bean.

Although it has often been assumed that polar interactions are of greatest importance in protein–carbohydrate complex formation, there has been very little evidence of substance to support this proposition. The existence of such polar-stabilizing forces for the binding of small neutral molecules to proteins has been suggested in recent years by several investigators (*cf.* Karush, 1957; Baker, 1967; Pollack *et al.*, 1967; Phillips, 1967).

The present study indicates that in the case of the α-methyl glycosides of D-glucose and D-mannose there exists a locus on the protein molecule which is involved in binding to the methoxyl function and which contributes to the total binding energy of the carbohydrate–concanavalin A complex. However, the presence of a C-1 β-methoxyl group represents a destabilizing factor in the binding of these glycosides to the active sites of the protein, probably through steric hindrance. This is demonstrated by the fact that 1,5-anhydro-D-glucitol and 1,5-anhydro-D-mannitol are poorer inhibitors than the respective methyl α-D-glycopyranosides of their parent sugars, but better inhibitors than the corresponding β-methyl glycosides.

The findings that methyl α-D-mannopyranoside was approximately three times more potent than the analogous sugar lacking the C-2 hydroxyl group (methyl 2-deoxy-α-D-*arabino*-hexopyranoside), and that a C-2 hydroxyl function of the D-*gluco* configuration (methyl α-D-glucopyranoside) produced a decrease in the inhibiting potency of the saccharide suggests that a C-2 hydroxyl group of the D-*manno* configuration contributes to the stabilization of the concanavalin A–carbohydrate complex; in the D-*gluco* configuration, however, a C-2 hydroxyl function represents a destabilizing factor.

TABLE V: Inhibiting Power of Aryl and Alkyl β-D-Glucopyranosides and β-D-Glucobioses.

Aryl or Alkyl β-D-Glucopyranoside	μmoles for 50% Inhibn
Methyl	37
Ethyl	37
n-Butyl	36
Isobutyl	35
Neopentyl	37
Benzyl	37
2′,2′,2′-Trifluoroethyl	58
Cyclopentyl	46
Cyclohexyl	65
Isopropyl	100
t-Butyl	165
Phenyl	7.0
Gentiobiose	55
Cellobiose	195 (7%)
Amygdalin	33
Phenyl β-cellobioside	195 (11%)

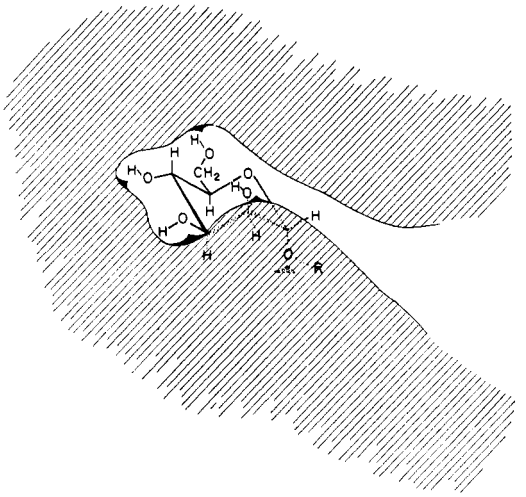

FIGURE 2: Diagrammatic representation of the polar saccharide binding site of concanavalin A.

The fact that D-mannose and its 2-*O*-methyl analog (as well as *p*-nitrophenyl α-D-mannopyranoside and its 2-*O*-methyl derivative) are equally effective as inhibitors of the concanavalin A system suggests that the *oxygen* atom (but not the hydrogen atom) of the C-2 position of D-mannose derivatives serves as a polar binding locus for the interaction of the saccharide with the protein. Moreover, replacement of the C-2 hydroxyl group of D-mannose by an acetamido function as in 2-acetamido-2-deoxy-D-mannose resulted in the formation of a very poor inhibitor. That this may not be due to steric interaction of the acetamido group with the protein is indicated by the relatively good inhibiting potency of 1,2-*O*-(1-methoxyethylidene)-β-D-mannopyranoside which contains a very bulky C-2 substituent.

The β-D-glucosidic oxygen atom may interfere with the binding of the saccharide to the protein since it was found that substitution of the glycosidic oxygen by sulfur caused a decrease in the inhibiting power of the molecule. These data indicate that the β-D-glycosidic oxygen lies extremely close to the protein resulting in a nonbonded interaction between this portion of the saccharide and the protein. This interaction is most evident from the very poor inhibitory power of β-D-glucopyranoside phenyl sulfone. Similarly the enhanced inhibiting power produced by the *p*-nitro group in the phenyl thio-D-glucopyranoside but not the *O*-glycoside series may be caused by the distortion of the electron distribution of the polarizable sulfur atom by the highly electron-withdrawing *p*-nitrophenyl function. Consistent with this concept, the ultraviolet absorption spectrum indicates that the B band of *p*-nitrophenyl 1-thio-β-D-glucopyranoside displays a large bathochromic shift (26 mμ) in relation to the B band of the spectrum of *p*-nitrophenyl β-D-glucopyranoside.

Examination of the C-2 derivatives of D-glucose also indicates a close contact between the protein and the hydroxyl group at the C-2 position. This is demonstrated by the low inhibiting potencies of the *O*-alkyl ethers of methyl α-D-glucopyranoside (such as the isopropyl and 2-cyanoethyl groups) as well as with the 2-acetamido-2-deoxy derivative. The protein is apparently not as close to this hydroxyl group as it is to the β-D-glycosidic oxygen atom of the bound saccharides since the inhibiting potencies of methyl α-D-glucopyranoside and its 2-*O*-methyl and 2-*O*-ethyl derivatives are comparable with those of methyl 2-deoxy-α-D-*arabino*-hexopyranoside (see Figure 2).

Direct participation of the C-3 hydroxyl group of both D-mannose and D-glucose is established by the fact that the 3-deoxy derivative of methyl α-D-mannopyranoside is 70 times less active as an inhibitor than the parent mannoside and the 3-deoxy derivative of phenyl β-D-glucopyranoside gave no inhibition at a level of 123 μmoles whereas 7 μmoles of the glucoside gave 50% inhibition.

Methyl 3-*O*-methyl α-D-glucopyranoside is 25 times less potent than methyl α-D-glucopyranoside (as compared with a 70-fold difference between the inhibiting power of methyl α-D-mannopyranoside and its 3-deoxy analog). This decrease in inhibiting power of the 3-*O*-methyl derivative is probably caused by steric factors, a conclusion substantiated by the fact that 3-*O*-benzyl-D-glucose is a noninhibitor of the concanavalin A–dextran system (Goldstein *et al.*, 1965).

Consistent with the apparent interaction of the C-3 *oxygen* atom with the protein is the finding that 3-deoxy-3-fluoro-D-glucose is only 1.5 times less potent an inhibitor of concanavalin A than is D-glucose, thus indicating that the electronegative fluorine atom may replace the hydroxyl function. This is in contrast to the observation that replacement of the C-6 hydroxyl group of methyl α-D-glucopyranoside by fluorine reduced the inhibition potency of this compound by at least 30-fold implicating the *hydrogen* atom of the C-6 hydroxyl group in hydrogen-bond formation with the protein (So and Goldstein, 1967a).

The very low inhibiting potency of methyl 4-deoxy-α-D-glucopyranoside indicates the importance of the C-4 hydroxyl group to the binding of the saccharide to concanavalin A. Goldstein *et al.* (1965) noted that D-galactose, the C-4 epimer of D-glucose is a noninhibitor of concanavalin A.

In general, it appears that polar interactions such as hydrogen bonds and charge–dipole interactions are the predominant stabilizing forces of concanavalin A–carbohydrate complexes. This is illustrated diagramatically in Figure 2. The polar binding loci involving the C-1, C-2, and C-3 oxygen atoms are denoted by the small triangular forms. The glycose is represented in the C-1 chair conformation, which is the normal conformation for most α- and β-D-glucopyranosides and D-mannopyranosides when in solution. Although there is no definitive evidence as to whether this is the conformation complementary to the binding site of concanavalin A, the fact that 1,2-*O*-(1-methoxyethylidene)-β-D-mannopyranoside (which appears from examination of Pauling–Corey–Koltum models to be incapable of existing in the 1C form) and 1-phenyl-1,5-anhydro-α-D-glucitol (which also appears to be most stable in the C-1 conformation) are excellent inhibitors of the concanavalin A–levan system, certainly supports the concept illustrated in Figure 2.

By comparing the inhibitory potency of a series of glycosides with methyl α-D-mannopyranoside whose association constant is known (So and Goldstein, 1968) it is possible to calculate the association constant for a number of the glycosides examined in this study. The association constant of each of these saccharides can be related to the free-energy change associated with binding to the protein by the relationship $\Delta F^\circ = -RT \ln K_a$. Table VI presents the equilibrium

TABLE VI: Binding Constants and Free-Energy Change Produced upon the Binding of Various Saccharides to Concanavalin A.

Compound	K_a	$\Delta F^{\circ\prime}$ (kcal/mole)[a]
Methyl α-D-mannopyranoside[b]	2.06×10^4	−5.4
Methyl α-D-glucopyranoside[c]	5.4×10^3	−4.7
1,5-Anhydro-D-glucitol[c]	9.8×10^2	−3.7
1,5-Anhydro-D-mannitol[c]	2.8×10^3	−4.3
Methyl 2-deoxy-α-D-*arabino*-hexopyranoside[c]	7.4×10^3	−4.9
Methyl 3-deoxy-α-D-*arabino*-hexopyranoside[c]	2.9×10^2	−3.1
Methyl 4-deoxy-α-D-*xylo*-hexopyranoside[c]	2.05×10^1	−1.6
Methyl 6-deoxy-α-D-gluco-pyranoside[c]	2.9×10^1	−1.8

[a] Calculated from relationship: $\Delta F^{\circ\prime} = -RT \ln K_a$. [b] K_a obtained by equilibrium dialysis. [c] Calculated from inhibitory potencies relative to methyl α-D-mannopyranoside (So and Goldstein, 1967a, 1968).

TABLE VII: $\Delta(\Delta F^{\circ\prime})$ Values for the Binding of Individual Binding Loci of Methyl α-D-Mannopyranoside to Concanavalin A.

Position	$\Delta F^{\circ\prime}_{\text{(parent sugar)}} - \Delta F^{\circ\prime}_{\text{(deoxy sugar)}}$	$\Delta(\Delta F^{\circ\prime})$ (kcal/mole)
C-1	Methyl α-D-mannopyranoside–1,5-anhydro-D-mannitol	−1.1
C-2	Methyl α-D-mannopyranoside–methyl α-deoxy-α-D-*arabino*-hexopyranoside	−0.5
C-3	Methyl α-D-mannopyranoside–methyl 3-deoxy-α-D-*arabino*-hexopyranoside	−2.3
C-4	Methyl α-D-glucopyranoside–methyl 4-deoxy-α-D-*xylo*-hexopyranoside	−3.1
C-6	Methyl α-D-glucopyranoside–methyl 6-deoxy-α-D-glucopyranoside	−2.9
Sum $\Delta(\Delta F^{\circ\prime})$		−9.9

constants and $\Delta F^{\circ\prime}$ values for the binding of some of these inhibitors.

By subtracting the $\Delta F^{\circ\prime}$ of the appropriate deoxy derivative from the $\Delta F^{\circ\prime}$ value of the parent compound, the free-energy change due solely to each hydroxyl group under consideration [$\Delta(\Delta F^{\circ\prime})$] may be calculated. Table VII lists the $\Delta(\Delta F^{\circ\prime})$ values for the contribution to binding to concanavalin A of each hydroxyl and methoxyl function of methyl α-D-mannopyranoside. The $\Delta F^{\circ\prime}$ values for methyl 4-deoxy-α-D-*xylo*-hexopyranoside and methyl 6-deoxy-α-D-glucopyranoside represent maximal values (these derivatives produced negligible inhibition at the highest concentration of inhibitor employed) and hence the $\Delta(\Delta F^{\circ\prime})$ terms for the C-4 and C-6 hydroxyl functions are minimal values and are probably somewhat greater than shown.

Summation of the $\Delta(\Delta F^{\circ\prime})$ values for the various hydroxyl groups of methyl α-D-mannopyranoside gives a value for the total free-energy change for the binding of an α-D-mannopyranoside residue to concanavalin A equal to −9.9 kcal/mole. However the experimentally determined value (So and Goldstein, 1968) is only −5.4 kcal/mole. Such a discrepancy between the calculated and observed free energy of binding of oligosaccharides to lysozyme was also noted by Chipman *et al.* (1967).

In regard to the topography of that portion of concanavalin A in contact with the aglycone of the bound glycoside, So and Goldstein (1967a) suggested that the low inhibiting potency of β-linked D-glucobioses, compared with the analogous α-linked disaccharides, may be due to steric hindrance caused by the nonbonded interaction of the reducing moiety of the saccharide with the protein. However, it is not solely the bulk of the aglycone which appears to be the determining factor. The inhibiting power of all the β-D-glucopyranosides of primary alcohols that were examined was virtually identical, regardless of the degree of substitution at the *β-carbon atom* of the aglycone. Those β-D-glucopyranosides which possess aglycones branched at the *α-carbon atom* displayed inhibiting powers inversely related to the degree of substitution at this carbon atom.

Since there is not any linear correlation between the Taft substituent constant (Taft, 1956) of the aglycone and the inhibiting potency of the saccharide, polar effects are apparently not involved. The absence of such a linear correlation is also consistent with the concept that the β-D-glucopyranosidic oxygen atom is not involved in the binding of the glycoside to concanavalin A.

In summary, the data strongly suggest that the O atoms of the C-1, C-2, and C-3 hydroxyl groups of α-D-mannopyranosyl residues participate in some type of noncovalent bonding with specific loci situated within the binding sites of the protein molecule. The essentiality of the C-4 hydroxyl group of the α-D-mannopyranosyl moiety has also been established although the precise atom involved in binding to the protein has not been identified.

Hydrogen-bonding forces almost certainly are involved (*cf.* Doyle *et al.*, 1968). The failure of high salt concentration (So and Goldstein, 1967b) to dissociate carbohydrate–concanavalin A complexes indicates that charge–charge interactions are probably not involved, a not surprising conclusion when it is considered that the carbohydrates studied were almost all neutral species.

The precise aminoacyl side chains of the concanavalin A molecule which are involved in binding to the carbohydrate moiety are currently being investigated. Preliminary titration and chemical modification studies suggest the involvement of protein side-chain carboxyl group (Hassing *et al.*, 1968).

Furthermore it appears that that part of the concanavalin A binding site in juxtaposition to the aglycone of the bound

saccharide is capable of accommodating aglycones of primary alcohols but only poorly those of secondary and tertiary alcohols. Consequently, the poorer inhibiting power of cellobiose as compared to gentiobiose may be due to non-bonded interactions of the protein with that portion of the reducing D-glucosido moiety adjacent to the glucosidic oxygen atom.

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