having a particle diameter of about 110 A, and the concentration of fraction I protein is 10-fold higher than the 70 and 80 S ribosomes having particle diameters of about 200 A, it may be difficult to recognize the latter by size alone. Disappearance of particles after RNAase treatment of the sections would not be conclusive evidence for ribosomes because fraction I protein particles might also be held in the mobile phase by binding to RNA and disappear when this material is hydrolyzed.

References

Clark, M. F., Matthews, R. E. F., and Ralph, R. K. (1963), *Biochem. Biophys. Res. Commun. 13*, 505.

Dorner, R. W., Kahn, A., and Wildman, S. G. (1957) *J. Biol. Chem. 229*, 945.

Eggman, L., Singer, S. J., and Wildman, S. G. (1953), *J. Biol. Chem. 205*, 969.

Francki, R. I. B., Boardman, N. K., and Wildman, S. G. (1965), *Biochemistry 4*, 865 (this issue; preceding paper).

Gierer, A. (1963), *J. Mol. Biol. 6*, 148.

Lyttleton, J. W. (1962), *Exptl. Cell Res. 26*, 312.

Marks, P. A., Burka, E. R., and Schlessinger, D. (1962), *Proc. Natl. Acad. Sci. U.S. 48*, 2163.

Spencer, D., and Wildman, S. G. (1964), *Biochemistry 3*, 954.

Warner, J. R., Knoff, P. M., and Rich, A. (1963), *Proc. Natl. Acad. Sci. U.S. 49*, 122.

Protein-Carbohydrate Interaction. II. Inhibition Studies on the Interaction of Concanavalin A with Polysaccharides*

I. J. Goldstein,† C. E. Hollerman,‡ and E. E. Smith

ABSTRACT: Concanavalin A, a lectin isolated from jack bean meal, has been reported to form a precipitate with certain types of ramified α-glucans such as glycogens, amylopectins, and dextrans, and α-mannans such as yeast mannan. This interaction is now shown to be amenable to investigation by the Landsteiner inhibition technique.

A large number of mono- and oligosaccharides and modified sugars have been studied for their capacity to inhibit the precipitation reaction between concanavalin A and a dextran. In this manner it has been possible to determine some of the stereochemical requirements of the combining sites of the concanavalin A molecule. Inhibition data suggest that unmodified hydroxyl groups at the C-3, C-4, and C-6 positions of the D-glucopyranose (or D-mannopyranose) ring are essential for binding to the active sites of the protein. A hydroxyl group at C-2 is not essential but the D-*manno* configuration is bound more firmly than the D-*gluco* configuration. The α-glycopyranosides of these sugars are much more active than the free sugars, whereas the corresponding β-glucosides are poor inhibitors. In like manner, the α-linked glucobioses are very tightly bound to the protein whereas the β-linked glucobioses that were tested are inactive. On a molar basis, the almost identical patterns of inhibition for maltose and maltotriose and for isomaltose and isomaltotriose further suggest that the interaction of concanavalin A with simple and complex saccharides is directed primarily toward the terminal, nonreducing portion of these molecules. These results suggest that the interaction of concanavalin A with polysaccharides involves the chain ends rather than intact inner branches, as has been suggested previously.

The specific interaction of protein with polysaccharide is well known in the field of immunology (Heidelberger, 1960). Thus, well documented studies have been reported on the immunochemical reactions of dextrans (Kabat, 1961; Sugg and Hehre, 1942), specific capsular polysaccharides of pneumococci (Heidelberger, 1956), and the *O*-antigens of *Salmonella* (Staub and Tinelli, 1957).

In addition to antibody proteins of higher animals, proteins are found in the seeds of certain plants which are capable of a similar type of interaction. These

* From the Department of Biochemistry, School of Medicine, State University of New York at Buffalo. *Received December 14, 1964.* This research was supported by U.S. Public Health research grants (AI-05542 and GM-09364) and by the United Health Fund of Buffalo.

† This work was done during the tenure of an Established Investigatorship of the American Heart Association. Present address: Department of Biological Chemistry, The Medical School, University of Michigan, Ann Arbor, Mich.

‡ Postdoctoral Fellow, Department of Pediatrics, Children's Hospital, State University of New York at Buffalo.

plant proteins demonstrate a specificity towards the agglutination of erythrocytes of various species, and Boyd and Shapleigh (1954) introduced the term "lectin" (Latin *legere*—to pick out or choose) in order to refer to their selectivity, yet differentiating them from immune antibodies. So specific are some of these lectins that they may be employed for the typing of blood in mammalian species (Boyd, 1963). In view of the known occurrence of mucopolysaccharides on the surface of erythrocytes and the demonstrated identity of these substances with the blood group determinants, it is an attractive hypothesis to explain the mechanism of action of lectins in terms of a combination with these carbohydrate determinants. Such an explanation was postulated by Sumner and Howell (1936) in their investigations into a phytohemagglutinin from the jack bean. This lectin, concanavalin A, was reported to agglutinate the erythrocytes of the dog, cat, rabbit, and guinea pig, but not of human, cow, goat, pig, sheep, or horse. In addition it was noted that concanavalin A was capable of agglutinating starch granules and of interacting to form an insoluble precipitate with glycogen and yeast mannan.

More recently (Cifonelli, *et al.*, 1956) this interaction was made the basis of a turbidimetric method for the quantitative estimation of glycogen. Under the conditions described it was noted that the extent of interaction was dependent on the source of the glycogen, and also that amylopectin, a molecule closely related to that of glycogen, failed to interact. Studies in this laboratory have shown that, under modified reaction conditions, not only amylopectin but also a large number of dextrans interact with concanavalin A (Goldstein *et al.*, 1965).

It was of interest therefore to study the concanavalin A interaction with a view to establishing the structural feature or features of the polysaccharides which permit combination at the receptor sites of the protein. The classical inhibition technique of Landsteiner (1920), which has proved so useful in immunochemical studies of carbohydrate-anticarbohydrate systems, has been applied in the present investigation. A large number of naturally occurring mono- and oligosaccharides and chemically modified sugars have been tested for their capacity to inhibit the precipitation reaction between concanavalin A and a dextran (NRRL B-1355-S, Jeanes *et al.*, 1954).

Materials and Methods

All sugars used in this study were examined for their purity by descending paper chromatography. The papers were eluted with an ethyl acetate–pyridine–water (10:4:3, v/v) solvent system (Aspinall and Ferrier, 1957) and spots developed with silver nitrate–sodium hydroxide spray reagent (Trevelyan *et al.*, 1950). When necessary, sugars were purified by preparative paper chromatography.

Sugar solutions of known concentration were prepared by weighing in the case of crystalline compounds, the crystals being previously dried to constant weight.

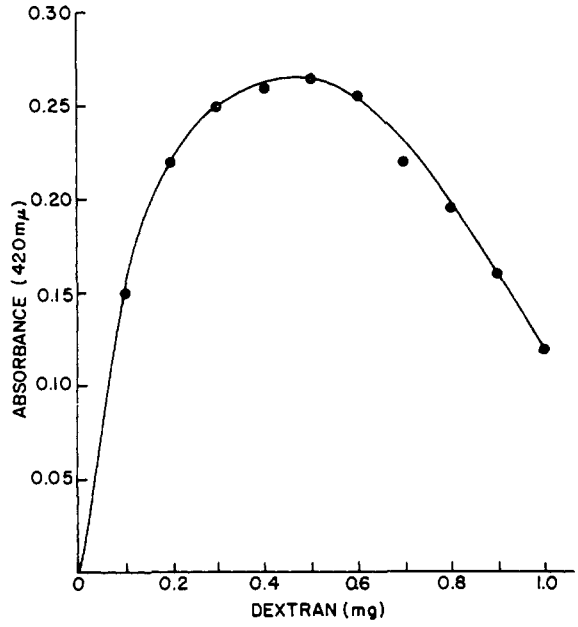

FIGURE 1: Relation between absorbance (420 mμ) and varying amounts of dextran (NRRL B-1355-S) in the presence of concanavalin A (0.84 mg total protein) in a total volume of 3.0 ml.

The concentrations of solutions of noncrystalline glucose-containing materials were determined by the phenol–sulfuric acid method (Dubois *et al.*, 1956).

Concanavalin A Solution. Concanavalin A was extracted from jack bean meal by the method of Sumner and Howell (1936). Purified preparations were dissolved in saturated NaCl solution and stored at 3°. For routine measurements of all inhibition assays the concanavalin A reagent contained the following components in a volume of 100 ml of one-half saturated NaCl solution: concanavalin A, 42 mg (determined as by Lowry *et al.*, 1951), and 0.1 mmole sodium acetate–hydrochloride buffer (*p*H 5.2).

Dextran Solution. Dextran (NRRL B-1355-S) was dissolved in water and the concentration was determined by the phenol–sulfuric acid method. The concentration was adjusted to 1.0 mg/ml by the addition of a suitable volume of water.

Precipitin and Inhibition Assays. Matched test tubes (10 × 1.3 cm) containing increasing amounts of dextran (NRRL B-1355-S) in water (1 ml) were preincubated at 25°. To each, 2 ml of concanavalin A solution (0.42 mg/ml) was added to a total volume of 3 ml, and the contents were stirred with a rod. After the solution had stood at 25° for eactly 10 minutes the absorbance at 420 mμ was measured in a Bausch and Lomb "Spectronic 20" spectrophotometer against a control test tube in which the dextran solution was replaced with water. Under these conditions no flocculation of the precipitates occurred within the 10-minute reaction time. A similar series of tubes in which the concanavalin A solution was replaced by 2 ml of half-saturated NaCl

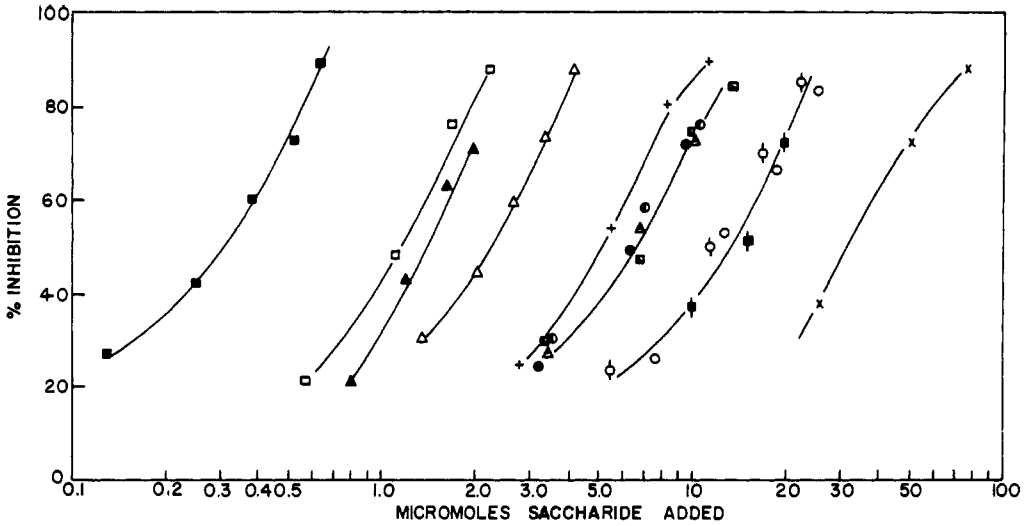

FIGURE 2: Inhibition of precipitation by monosaccharides and their derivatives. ×, methyl β-D-glucopyranoside; ▮, L-sorbose; ○, *N*-acetyl-D-glucosamine; ◇, D-glucose; ◧, 2-deoxy-1,5-anhydro-D-*arabino*-hexitol; ◐, 1,5-anhydro-D-glucitol; △, 2-deoxy-D-glucose; ●, 2-*O*-methyl-D-glucose; +, D-fructose; △, D-mannose; ▲, methyl α-L-sorbopyranoside; □, methyl α-D-glucopyranoside; ■, methyl α-D-mannopyranoside. Reaction mixture contains 0.50 mg dextran (NRRL B-1355-S) and 0.84 mg total protein (concanavalin A extract) in a volume of 3.0 ml.

solution was prepared and measured in a similar manner. The difference between these readings and the absorbances of the corresponding test solutions was plotted against the weight of dextran present to give the equivalence curve shown in Figure 1. The reaction mixture containing 0.50 mg of dextran exhibits a reading close to maximum absorbance and was selected to carry out the inhibition assays.

An initial screening for inhibitory power was carried out by including relatively large amounts of each sugar ($\geqslant 20$ μmoles) and 0.50 mg of dextran in a total volume of 1.0 ml. Concanavalin A solution (2 ml, 0.42 mg/ml) was added and the absorbance was determined as before. Those sugars which produced a significantly lower absorbance after 10 minutes were classed as inhibitors and were tested so as to give a wide range of inhibition of the precipitation reaction.

Percentage inhibition was calculated from the expression $(A - B)/A \times 100$ where A and B represent the absorbance, corrected for the dextran absorbance, obtained in the absence and presence of inhibitor, respectively. Inhibition curves were constructed by plotting the calculated values for percentage inhibition versus the common logarithm of the μmoles of inhibitor added.

Results

A number of the sugars tested exhibited an extremely small but finite inhibition of the interaction. We have arbitrarily classed as noninhibitors those sugars which, when added to the test system in an amount equivalent to 20 μmoles, caused less than 10% inhibition of precipitation. A list of noninhibitors is presented in Table I. These include all of the pentoses and polyhydric alcohols tested.

D-Mannose, D-fructose, D-glucose, and L-sorbose, in that order of potency, are the only monosaccharides tested which exhibited a significant inhibition of the interaction (Figure 2). D-Glucose, although a relatively poor inhibitor (50% inhibition by 11 μmoles), apparently contains at least a portion of the configuration required by the receptor sites of concanavalin A. It can readily be seen from Table I that substitution or modification of any one of the hydroxyl groups at C-3, C-4, or C-6 results in a complete loss of activity.

The identical curves of inhibition obtained for 2-deoxy-D-glucose and 2-*O*-methyl-D-glucose (Figure 2) indicate a slightly greater activity than that of D-glucose, whereas *N*-acetyl-D-glucosamine is, within the bounds of experimental error, equally as good an inhibitor as D-glucose. This seems to indicate that the C-2 hydroxyl group is not essential for binding to the protein. The inhibition curves for 1,5-anhydro-D-glucitol and 2-deoxy-1,5-anhydro-D-*arabino*-hexitol, lying on the same curve as 2-*O*-methyl- and 2-deoxy-D-glucose, suggest that the hydroxyl group at C-1 also is not involved in binding. However, that this is not the case is shown by the approximately 10-fold increase in activity (relative to D-glucose) of methyl α-D-glucopyranoside (1.1 μmoles for 50% inhibition) and by the very low potency of methyl β-D-glucopyranoside (33 μmoles for 50% inhibition) which falls to a value of only one-third that of D-glucose.

The inhibition by D-mannose and its derivatives roughly parallels the pattern found for D-glucose and its derivatives. Although no D-mannose derivative modified at the C-4 hydroxyl group has been tested, it

TABLE I: Substances Tested for Inhibition Activity.

Inhibitors	Noninhibitors	Other Noninhibitors	
Active Hexoses and Their Derivatives		**Hexoses and Derivatives**	
D-Glucose			
1,5-Anhydro-D-glucitol	3-Deoxy-D-glucose	D-Allose	L-Glucose
2-Deoxy-1,5-anhydro-D-*arabino*-hexitol	3-*O*-Methyl-D-glucose	D-Altrose	D-Gulose
2-Deoxy-D-glucose	3-*O*-Benzyl-D-glucose	D-Galactose	L-Mannose
2-*O*-Methyl-D-glucose	4-Deoxy-D-glucose	L-Galactose	L-Rhamnose
N-Acetyl-D-glucosamine	4-*O*-Benzyl-D-glucose	1,6-Anhydro-β-D-galacto-pyranose	D-Talose
Methyl α-D-glucopyranoside	6-*O*-Methyl-D-glucose	L-Fucose	D-Tagatose
Methyl β-D-glucopyranoside[a]	1,6-Anhydro-β-D-gluco-pyranose	*N*-Acetyl-D-galactosamine	
	D-Glucuronolactone	**Pentoses and Derivatives**	
	D-Sorbitol	D-Arabinose	D-Lyxose
	Ethyl β-D-glucofuranoside	D-Arabinitol	D-Ribose
	1,2-*O*-Isopropylidine-D-glucofuranose	L-Arabinose	D-Xylose
D-Mannose		L-Arabinitol	Methyl α-D-xylo-pyranoside
Methyl α-D-mannopyranoside	*N*-Acetyl-D-mannosamine	**Miscellaneous**	
	3-Deoxy-D-mannose	Glycine	
	1,6-Anhydro-β-D-manno-pyranose	Ethylene glycol	
D-Fructose		Glycerol	
L-Sorbose	6-Deoxy-D-mannose	Erythritol	
Methyl α-L-sorbopyranoside	D-Mannitol	Myoinositol	
Oligosaccharides		2-*O*-Methylmyoinositol	
Maltose	Cellobiose	2,6-Anhydro-D-*glycero*-D-*gulo*-heptitol	
Isomaltose	Gentiobiose	2,6-Anhydro-D-*glycero*-D-*manno*-heptitol	
Nigerose	Laminaribiose	Cyclohexanecarbinol	
Kojibiose		*cis*-2-Hydroxycyclohexanecarbinol	
α,α-Trehalose	Melibiose	*cis*-3-Hydroxycyclohexanecarbinol	
Sucrose		Phloroglucinol	
Turanose	Lactose	Hydroquinone	
3-*O*-α-D-Glucopyranosyl-D-arabinose			
Maltotriose	Raffinose		
Isomaltotriose			
Panose			
Melezitose			

[a] Relatively poor inhibitor.

can be seen that modification at C-3 or C-6 (3-deoxy- or 6-deoxy-D-mannose) similarly results in a complete loss of activity (Table I). As in the case of D-glucose the presence of an α-methyl glycosidic linkage confers on the molecule a very large increase in activity, such that methyl α-D-mannopyranoside is the most potent of all the inhibitors tested (Figure 2). Unlike D-glucose, however, the *N*-acetylamido derivate of D-mannose (2-acetamido-2-deoxy-D-mannose) is a noninhibitor.

Of the disaccharides tested only those containing the α-glycosidic bond were active. The inhibition curves obtained are shown in Figure 3. It is apparent that for those disaccharides containing only D-glucose, the type of α-glucosidic linkage present is a factor influencing their activity as inhibitors. Isomaltose (α-1,6- linkage) and α,α-trehalose (α,α-1,1- linkage) are equally potent, approximately 1.1 μmoles of either being required for 50% inhibition. These disaccharides are approximately ten times more active than the monosaccharide. Rather less active, although still superior to glucose, are kojibiose (α-1,2- linkage), maltose (α-1,4- linkage), and nigerose (α-1,3- linkage), in order of decreasing activity. The β- isomers of three of these disaccharides, viz., gentiobiose (β-1,6- linkage), cellobiose (β-1,4- linkage), and laminaribiose (β-1,3- linkage), are all noninhibitors (Table I). Although the inhibition curve of turanose, a disaccharide in which glucose is linked through an α-1,3- bond to D-fructose (3-*O*-α-D-glucopyranosyl-D-fructose), lies very near to that of nigerose (Figure 3), it is evident that sucrose

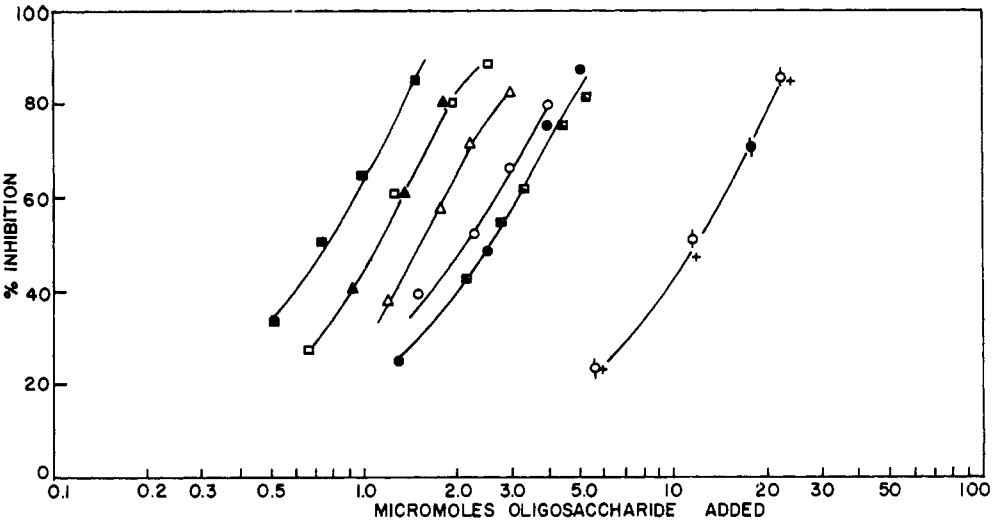

FIGURE 3: Inhibition of precipitation by oligosaccharides. ♀, D-glucose; +, sucrose; ◪, nigerose; ●, turanose; ○, maltose; △, kojibiose; □, isomaltose; ▲, α,α-trehalose; ■, 3-*O*-α-D-glucopyranosyl-D-arabinose. Reaction mixture contains 0.50 mg dextran (NRRL B-1355-S) and 0.84 mg total protein (concanavalin A extract) in a volume of 3.0 ml.

(2-*O*-α-D-glucopyranosyl-β-D-fructofuranoside), which contains an α-1,2-glucosidic linkage, is inferior to kojibiose (α-1,2-glucobiose), being comparable in activity to glucose. It is evident, therefore, that the nature of the aglycone, in addition to the type of α-glycosidic linkage present, is important. This is further supported by the very high potency of 3-*O*-α-D-glucopyranosyl-D-arabinose. The presence of D-arabinose at the reducing end of this disaccharide, rather than D-glucose as in nigerose, increases its inhibition activity by a factor greater than three.

Examination of the structures of the inhibiting disaccharides reveals that each possesses an unsubstituted α-D-glucopyranosyl unit at its nonreducing end. The possibility that disaccharides, which contain a D-mannopyranosyl residue in this position, may also act as inhibitors remains a matter of speculation until such compounds are tested. It is significant, in the light of the observed inactivity of D-galactose, that three oligosaccharides containing an unsubstituted D-galactopyranosyl residue at their nonreducing end also failed to inhibit. In the case of lactose (4-*O*-β-D-galactopyranosyl-D-glucose) this effect is not definitive as, in addition to the nonreducing galactose residue, the glycosidic linkage of this disaccharide has the β- configuration. However, melibiose (6-*O*-α-D-galactopyranosyl-D-glucose) contains an α-1,6- bond but still fails to exhibit any significant inhibition. The trisaccharide raffinose (*O*-α-D-galactopyranosyl-(1→6)-*O*-α-D-glucopyranosyl-(1→2)-β-D-fructofuranoside) has a melibiose moiety at one end and, as expected, also failed to inhibit the concanavalin A interaction. This trisaccharide can be considered as a sucrose derivative in which an α-D-galactopyranosyl residue has been linked to the C-6 hydroxyl group of the D-glucose unit. Attachment of an α-D-glucopyranosyl residue to the D-fructose moiety gives rise to a second trisaccharide, melezitose (*O*-α-D-glucopyranosyl-(1→3)-*O*-β-D-fructofuranosyl-(2→1)-*O*-α-D-glucopyranoside). This trisaccharide is approximately thirty times more potent than sucrose, exhibiting an activity nearly as great as methyl α-D-mannopyranoside.

In like manner isomaltotriose and maltotriose can be considered as arising from substitution of an α-D-glucopyranosyl unit at the C-6 hydroxyl group of the nonreducing moiety of isomaltose and at the C-4 hydroxyl group of the nonreducing moiety of maltose, respectively. In neither case, however, was a change in inhibition observed from that of the parent disaccharide (Figure 4). The inhibition pattern of the trisaccharide, panose (*O*-α-D-glucopyranosyl-(1→6)-*O*-α-D-glucopyranosyl-(1→4)-D-glucose) also lies on the same curve as that of the isomaltose series and it is significant that these three oligosaccharides have an identical structure at their nonreducing end.

Discussion

Landsteiner's inhibition reaction is based upon the concept that a small, relatively simple compound (hapten), which possesses an identical or related structure to that of a specific determinant on an antigen, can also interact with the corresponding antibody. Haptens having only one reactive group do not precipitate with antibody but can effectively compete with antigen for the receptor sites on an antibody molecule and, as a consequence, inhibit the precipitin reaction between antibody and antigen. The extent of inhibition is related to the affinity of the hapten for the antibody receptor sites. A knowledge of the relative inhibiting

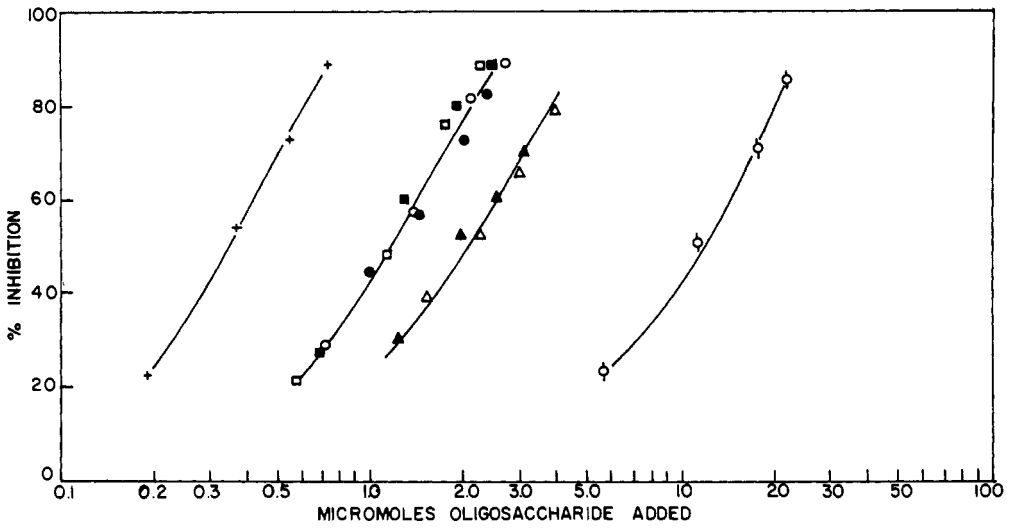

FIGURE 4: Inhibition of precipitation by oligosaccharides. ○, D-glucose; △, maltose; ▲, maltotriose; ■, isomaltose; ●, isomaltotriose; O, panose; □, methyl α-D-glucopyranoside; +, melezitose. Reaction mixture contains 0.50 mg dextran (NRRL B-1355-S) and 0.84 mg total protein (concanavalin A extract) in a volume of 3.0 ml.

power of haptens of known molecular structure, therefore, provides an extremely valuable tool in determining the stereochemical requirements of the antibody receptor sites. This technique, which has been so useful in the immunochemical study of carbohydrate-anti-carbohydrate systems, has been applied in the present study to the interaction of dextran with concanavalin A.

The observed interaction of concanavalin A with four different monosaccharides (D-glucose, D-mannose, D-fructose, and L-sorbose) does not necessarily imply the presence of four receptor sites on the protein, inasmuch as a configurational feature held in common by the monosaccharides may be sufficient to satisfy the specific requirements of a single receptor site. This type of interaction, in which the specific requirement of the interacting protein is directed toward only a portion of the sugar molecule, is well known, and has been used for a classification of monosaccharides with respect to their specific inhibiting activity for plant agglutinins (Mäkelä, 1957).

Two of the four inhibiting monosaccharides, D-glucose and D-mannose, exist in solution and occur in polysaccharides predominantly in the pyranose ring form. That it is this ring form which participates in the interaction with concanavalin A is indicated by the high inhibition activity of their corresponding methyl α-glycopyranosides and also by the failure of the open-chain hexitols, D-sorbitol and D-mannitol, and of ethyl β-D-glucofuranoside to exhibit any inhibition activity.

Of the four possible ring forms of D-fructose (α- and β-furanose and pyranose) there are three which may be of significance in binding to concanavalin A. A pyranose form (β-D-fructopyranose) has hydroxyl groups at C-1, C-3, C-4, and C-5 which can be related to the C-2, C-3, C-4, and C-6 hydroxyl groups of D-mannopyranose. The hydroxyl groups at C-3, C-4, and C-6 of both the α- and β-furanose forms are identical, in terms of their configuration, to the hydroxyl groups at the corresponding carbon atoms of D-glucopyranose. In addition, β-D-fructofuranose has hydroxyl groups at C-1, C-3, and C-4, which can be similarly related to those at C-3, C-4, and C-6 of D-glucopyranose. A definite decision as to which form (or forms) binds to concanavalin A must await further experimentation. Similar considerations suggest that α-L-sorbopyranose, which can be related to D-glucopyranose, may be the form which is active as an inhibitor. The finding that methyl α-L-sorbopyranoside is a good inhibitor (Figure 2) seems to support this conclusion.

Our results further indicate that it is the unmodified hydroxyl groups at C-3, C-4, and C-6 of the D-glucopyranosyl or D-mannopyranosyl ring forms which are involved in binding to the active sites of the protein molecule. The absolute requirement for these configurational features is evidenced by the almost complete lack of inhibition by the corresponding C-3 and C-4 epimers of D-mannose and of D-glucose (D-altrose and D-talose, and D-allose and D-galactose, respectively; Table I) and by the inactivity of all those D-glucose and D-mannose derivatives tested, in which chemical modification has been effected at C-3, C-4, or C-6 (Table I).

The demonstration that 2-deoxy-D-glucose, 2-*O*-methyl-D-glucose, 1,5-anhydro-D-glucitol, and 2-deoxy-1,5-anhydro-D-*arabino*-hexitol exhibit little or no change in inhibition activity over that of the parent D-glucose suggests that hydroxyl groups at the C-1 and C-2 positions are not essential for protein interaction. Indeed, the last-named substance is the simplest compound tested which still possesses the required configurational features necessary for binding to the protein.

That some participation by the C-2 hydroxyl group occurs is suggested by the observation that D-mannose and its methyl α-glycopyranoside are approximately three and five times more active, respectively, than D-glucose and its corresponding α-glycoside. Moreover, the failure of *N*-acetyl-D-mannosamine to inhibit the precipitation reaction may indicate that the C-2 hydroxyl group in the D-*manno*- configuration more closely approaches the surface of the protein than in the D-*gluco*- configuration. Hence the presence of the relatively bulky acetamido group would be expected to introduce a larger steric effect in the D-mannose derivative than in *N*-acetyl-D-glucosamine, which possesses an inhibition activity essentially unchanged from that of D-glucose.

It is apparent from the high inhibition activity exhibited by the methyl α-glycopyranosides of D-glucose and D-mannose that, in addition to the essential requirement for the D-*arabino*- configuration at C-3, C-4, and C-5, the combining sites of concanavalin A have a high degree of specificity for the α- configuration at the anomeric carbon atom. This receives support from the low activity of methyl β-D-glucopyranoside (33.0 μmoles for 50% inhibition) relative to that of the α-glycoside (1.1 μmoles for 50% inhibition) and from the results of Goldstein *et al.* (1965) in which it was reported that, of the large number of glucans and mannans tested, only those containing α-glycosidic linkages showed a positive interaction with concanavalin A (dextrans, glycogen, amylopectin, and yeast mannan). The β-linked polysaccharides (laminarin, oat gum glucan, lichenin, and the mannans of ivory nut and salep) failed to interact. The further observation that yeast mannan interacted more strongly than twelve of the sixteen α-glucans capable of reacting with concanavalin A may be related to the higher activity of the D-mannose compounds observed in our inhibition studies.

The importance of the α- configuration at C-1 is further confirmed by the high potency, relative to glucose, of the α-linked glucose disaccharides (Figure 3) and by the inactivity of the corresponding β-linked disaccharides which were available for testing (laminaribiose, cellobiose, and gentiobiose). The close proximity of the inhibition curves of five α-linked glucose disaccharides indicates that concanavalin A does not exhibit a high degree of specificity toward any one type of α-glucosidic bond. The coincidence of the inhibition curve for isomaltose (α-1,6- linkage) with that of methyl α-D-glucopyranoside makes it unlikely that the reducing glucose unit in this disaccharide participates actively in the binding mechanism. The relatively small differences in the binding affinities of the α-linked glucobioses may well be interpreted in terms of a steric effect. Thus isomaltose, in which two D-glucose molecules are glycosidically united by an α-1,6- bond, may allow a closer approach to the surface of the protein molecule than if they possess an α-1,2-glucosidic linkage, as in kojibiose. This is assuming, as this study strongly indicates, that it is primarily the terminal, nonreducing sugar unit which is involved in binding to the concanavalin A molecule. In the same manner an α-1,2-glucosidic linkage may give rise to greater binding than an α-1,4-glucosidic bond, which in turn might allow a closer approach to the protein surface than an α-1,3-glucosidic linkage.

Addition of a third similarly linked glucose unit to either isomaltose or maltose results in no change in binding affinity as is shown by the fact that the corresponding trisaccharides, isomaltotriose and maltotriose, respectively, exhibit no increase in activity on a molar basis (Figure 4). Panose, which can be considered as an isomaltose molecule glucosidically linked to C-4 of a third glucose molecule, also exhibits an activity equal to that of isomaltose. This suggests that the α-1,4- bond is sufficiently remote from the nonreducing end of the molecule to have little or no effect on its binding affinity. These results indicate that in both the maltose and isomaltose series the effective binding of the molecule is probably restricted to the nonreducing glucopyranosyl residue and is affected only by the nature of the bond linking it to the remainder of the molecule. The wide variation in inhibitory power of oligosaccharides which contain sugars other than D-glucose (e.g., sucrose and turanose) is not completely understood and further studies are required in place of conjecture.

Both melezitose and α,α-trehalose are excellent inhibitors of the concanavalin A–dextran precipitation reaction. Each contains two terminal, nonreducing α-D-glucopyranosyl residues. The exceptionally high potency of melezitose may well indicate that the two α-D-glucopyranosyl residues in this molecule are each capable of occupying active sites on the concanavalin A molecule without significant steric hindrance by the other.

All the inhibitors and noninhibitors reported here for the dextran–concanavalin A interaction have been examined for their molar inhibitory activity in the interaction of concanavalin A with both glycogen and yeast mannan. The relative activities of the inhibitors were found to be similar in both systems and to closely parallel the inhibition pattern described in the present study. This provides further evidence that identical receptor sites on the protein are probably responsible for the interaction of concanavalin A with these polysaccharides.

Although further inhibition studies are in progress, we feel that the present results allow some general conclusions to be drawn with regard to the mechanism of the interaction of concanavalin A with α-glucans. It has been suggested by Cifonelli *et al.* (1956) that the receptor sites of concanavalin A are directed toward the intact, inner branches of glycogen. The α-D-glucopyranosyl residues of the inner branches of glycogen, however, are, of necessity, involved in linkage at C-6 and/or C-4, which, on the basis of our inhibition studies, would result in their having no affinity toward the protein receptor sites. The α-D-glucopyranosyl residues at the nonreducing chain ends of the glycogen molecule, however, are involved in linkage at C-1 only and therefore possess the required configuration

for interaction with concanavalin A. The large number of such interacting chain ends in the highly branched glycogen molecule may be sufficient to account for the precipitation reaction observed with concanavalin A. The approximately direct relationship reported between the extent of interaction with concanavalin A and the degree of branching of glycogens (Manners and Wright, 1962) and of dextrans (Goldstein *et al.*, 1965) can be readily explained on the basis of a chain-end interaction, in that the number of chain ends is directly related to the degree of branching of the polysaccharide molecule.

The report that amylopectin will form a precipitate with concanavalin A (Smith and Goldstein, 1964; Goldstein *et al.* 1965) is also consistent with the concept of outer-chain interaction. More recent observations in this laboratory (unpublished results) indicate that factors such as protein concentration and molecular size of the polysaccharide offer an explanation for the failure of amylopectin to precipitate under the conditions used by Cifonelli *et al.* (1956) and Manners and Wright (1962).

This concept of chain-end interaction is analogous to the mechanism of interaction of immune antibodies with their corresponding antigenic dextrans. Anti-dextran proteins have been shown to interact at the nonreducing chain ends of the dextran molecule but, in contrast to concanavalin A, they generally exhibit a high degree of specificity toward linkage type and require for maximal activity a fragment of the chain end consisting of six or seven α-D-glucopyranosyl residues (Kabat, 1961). As a result of this high specificity the number of dextrans capable of interaction with any one immune antibody is strictly limited. The apparent requirement of concanavalin A for only a single α-D-glucopyranosyl residue to give maximal interaction is therefore in accord with the large number of naturally occurring polysaccharides with which it has been shown to interact.

Acknowledgments

The technical assistance of Miss Eileen Heffernan and Mr. Harrison Sine is acknowledged. We thank the following for gifts of carbohydrate specimens: Prof. L. Anderson, Dr. R. de Souza, Dr. H. G. Fletcher, Jr., Dr. H. S. Isbell, Prof. K. Matsuda, Prof. W. G. Overend, Dr. A. S. Perlin, Dr. J. Prokop, Dr. N. K. Richtmyer, Prof. S. Roseman, Prof. H. J. Schaeffer, and Prof. W. J. Whelan.

References

Aspinall, G. O., and Ferrier, R. J. (1957), *J. Chem. Soc.*, 4188.

Boyd, W. C. (1963), *Vox Sanguinis 8*, 1.

Boyd, W. C., and Shapleigh, E. (1954), *Science 119*, 419.

Cifonelli, J. A., Montgomery, R., and Smith, F. (1956), *J. Am. Chem. Soc. 78*, 2485.

Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A., and Smith, F. (1956), *Anal. Chem. 28*, 350.

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

Heidelberger, M. (1956), Lectures in Immunochemistry, New York, Academic.

Heidelberger, M. (1960), *Fortschr. Chem. Org. Naturstoffe 18*, 503.

Jeanes, A., Haynes, W. C., Wilham, C. A., Rankin, J. C., Melvin, E. H., Austin, M. J., Cluskey, J. E., Fischer, B. E., Tsuchiya, H. M., and Rist, C. E. (1954), *J. Am. Chem. Soc. 76*, 5041.

Kabat, E. A. (1961), *in* Kabat and Mayer's Experimental Immunochemistry, 2nd ed., Springfield, Ill., C. C Thomas.

Landsteiner, K. (1920), *Biochem. Z. 104*, 280.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem. 193*, 265.

Mäkelä, O. (1957), Studies in Hemagglutinins of Leguminosae Seeds, Helsinki, Weilin and Göös.

Manners, D. J., and Wright, A. (1962), *J. Chem. Soc.*, 4592.

Smith, E. E., and Goldstein, I. J. (1964), Abstracts of Papers, 148th Meeting, American Chemical Society, Chicago, Aug.-Sept. 1964, p. 29D.

Staub, A. M., and Tinelli, R. (1957), *Bull. Soc. Chim. Biol., Suppl. 1*, 65.

Sugg, J. Y., and Hehre, E. J. (1942), *J. Immunol. 43*, 119.

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

Trevelyan, W. E., Proctor, D. P., and Harrison, J. S. (1950), *Nature 166*, 444.