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# PROTEIN–CARBOHYDRATE INTERACTION

## PART XXI. THE INTERACTION OF CONCANAVALIN A WITH D-FRUCTANS*

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ABSTRACT

The immunochemical techniques of quantitative precipitation and hapten inhibition have been employed in a study of the interaction of concanavalin A with several levans of bacterial and plant origin. Of the polysaccharides that form a precipitate with concanavalin A (dextrans, D-mannans, glycogens, amylopectins, and levans), levans appear to be the least reactive.

Hapten-inhibition studies were conducted with a wide variety of compounds containing a furanoid ring. As a generalization, it may now be stated that all sugars containing the 1,4-anhydro-D-arabinitol moiety can bind to the active sites of concanavalin A. Because evidence from this laboratory suggests that the interaction of concanavalin A with reactive polysaccharides occurs at the same binding sites of the protein, reaction with levans may be rationalized on the basis of common configurational features. The disposition of the hydroxyl groups on C-3, C-4, and C-6 (or C-1, C-3, and C-4) of the β-D-fructofuranosyl moiety, which is the residue that occurs at chain ends of levans, is similar to that on C-3, C-4, and C-6 of the α-D-glucopyranosyl moiety, the sugar residue found at the chain ends of α-D-glucans.

## INTRODUCTION

In earlier studies, concanavalin A was shown to react only with polysaccharides containing D-glucose and D-mannose[1–3]. The first demonstration of the interaction of concanavalin A with levans was reported by Goldstein and So[4,5], who used the Ouchterlony, double-diffusion technique. Recently, it was reported[6] that the levan from *L. mesenteroides* strain C also reacts to form a precipitate with concanavalin A. Levans are high molecular-weight, branched-chain D-fructans, elaborated in the growth media of certain micro-organisms, in which the β-D-fructofuranosyl residues are joined via (2→6) glycosidic linkages, with branch points originating from the

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hydroxymethyl groups of every 9–12 D-fructofuranosyl residues. Levans of plant origin are also known[7].

In the present study, the immunochemical techniques of quantitative precipitation[8] and hapten inhibition[9,10] (see Kabat[11] for an extended discussion of these techniques) have been employed in an examination of levan–concanavalin A interaction. To establish a basis for this interaction, a variety of sugars containing D-fructofuranosyl residues was examined for their capacity to inhibit the concanavalin A–polysaccharide interaction.

## MATERIALS AND METHODS

*Aerobacter levanicum* No. 15552 was obtained from the American Type Culture Collection. *Bacillus megatherium* KM was a gift from Dr. J. M. Merrick. Dextran NRRL B-1355-S and the levan from *Bacillus subtilis* were generous gifts from Drs. Allene R. Jeanes and D. Feingold, respectively. A D-gluco-D-fructan from the Hawaiian Ti plant was obtained from Dr. B. Lewis. Planteose was a gift from Dr. D. French; the methyl ethers of D-fructose were provided by Dr. Elizabeth E. Percival; allosucrose was a gift from Dr. D. Feingold; the 1,4-anhydropentitols and 2,5-anhydro-L-iditol and 2,5-anhydro-D-mannitol were provided by Dr. R. Barker; 2,5-anhydro-D-glucitol was presented by Dr. J. W. LeMaistre; 1,5-anhydro-D-glucitol and 1,5-anhydro-D-mannitol were the gift of Dr. N. K. Richtmyer; 1-kestose and nystose were given to us by Dr. W. W. Binkley; Dr. C. T. Bishop provided the methyl pentofuranosides; and Dr. B. Lewis, di-D-fructose 1,2′:2,1′-dianhydride. Inulobiose and inulotriose were isolated from a partial, acid-hydrolyzate of inulin (Mann Research Laboratories, Inc., New York) by descending paper-chromatography with butyl alcohol–ethanol–water [4:1:5 (v/v), upper phase] under the conditions of Pazur and Gordon[12]. Planteobiose was isolated from planteose according to the procedure of French *et al.*[13]. Mr. Gordon Hassing of this laboratory provided samples of the four methyl glycosides of D-fructose.

Concanavalin A was obtained in a highly purified state by the Sephadex method of Agrawal and Goldstein[14].

Cross-linked, levan gel was provided by Dr. E. A. Kabat.

*Growth of bacteria.* — Stock cultures of *A. levanicum* were maintained on nutrient-agar slants (Difco Laboratories, Detroit, Michigan), and grown in nutrient broth (Difco Laboratories) containing sucrose, as described by Hestrin *et al.*[15]. Sucrose solutions were sterilized by filtration through a bacterial, ultra-fine filter (E. H. Sargent and Co.).

Stock cultures of *Bacillus megatherium* KM were maintained on 1% yeast extract in 1.5% agar. Cells were grown in the presence of sucrose. The growth medium (personal communication, Dr. J. M. Merrick) contained 266.6 ml of 0.1M $Na_2HPO_4$, 133.3 ml of 0.1M $KH_2PO_4$, 3.0 g of NaCl, 1.0 g of $NH_4Cl$, 0.1 g of $Na_2SO_4$, 0.1 g of $MgCl_2 \cdot 6\,H_2O$, 0.01 g of $MnCl_2 \cdot 4\,H_2O$, and 0.1 g casamino acid (vitamin-free) per liter of solution.

*Isolation of levan.* — After growth for 24 h at 30°, cells were harvested by centrifugation at 8,000 r.p.m. for one h in a Sorvall RC-2 centrifuge equipped with a GSA head. Levan was precipitated from the supernatant solution by the addition of approximately three volumes of 95% ethanol; addition of a few drops of 1% calcium chloride solution facilitated flocculation[15]. The gummy precipitate that separated was triturated with absolute methanol. Purification was achieved by dissolution in warm water, filtration, reprecipitation with ethanol, and trituration with methanol. Deproteinization with one-third of the volume of 3:1 (v/v) chloroform–1-octanol was effected by the Sevag procedure[16].

Complete hydrolysis, with acid, of levans isolated from the growth media of *A. levanicum* and *B. megatherium* gave fructose as the sole constituent monosaccharide on paper-chromatographic analysis. Hydrolysis was conducted by heating a solution of the levan in 5% aqueous oxalic acid in a sealed tube for one h in a boiling-water bath. The solution was made neutral with solid calcium carbonate, the suspension was filtered, and the filtrate was concentrated *in vacuo* at 40°. Paper chromatography was performed by the descending technique, with butyl alcohol–ethanol–water [4:1:5 (v/v), upper phase] as the irrigating solvent. After being dried, paper chromatograms were sprayed with *p*-anisidine[17].

*Quantitative precipitation.* — Studies on quantitative precipitation were performed as described by So and Goldstein[8]. Increasing amounts of polysaccharide were added to a series of tubes containing a constant amount of concanavalin A. A typical incubation mixture consisted of the following components: concanavalin A in M NaCl (0.1 ml), 4.1M NaCl (0.22 ml), 0.1M phosphate buffer (pH 7.2, 0.18 ml), and polysaccharide in water (0.5 ml).

The amount of protein nitrogen in the precipitates was determined as previously described[8], by ninhydrin analysis of the washed precipitates, which had been digested with 3.5M sulfuric acid.

*Carbohydrate analysis of precipitates.* — The carbohydrate composition of concanavalin A–levan precipitates was determined by the phenol–sulfuric acid method[18], performed directly on the protein–polysaccharide precipitates, as previously described[8]. The total amount of polysaccharide in the precipitate was determined by reference to a standard curve for D-fructose, the conversion to give the value for polysaccharide being made by multiplying by 0.9.

The concentration of all solutions of polysaccharide, as well as those of sugars employed in hapten inhibition, was determined by the phenol–sulfuric acid method[18].

*Hapten inhibition.* — Experiments on hapten inhibition were conducted by determining the extent to which various carbohydrates of low molecular weight inhibited concanavalin A–dextran precipitation[10]. A typical, incubation mixture employed in these studies contained the following components: concanavalin A (300 μg) in M NaCl (0.1 ml), 4.1M NaCl (0.22 ml), 0.1M phosphate buffer (pH 7.2, 0.18 ml), dextran B-1355-S (200 μg, 0.1 ml), and inhibitor in water (0.4 ml). When the concanavalin A–levan (*A. levanicum*) system was employed in hapten-inhibition studies, 100 μg of levan was used in quantitative precipitation, and analysis for protein

nitrogen was conducted as described earlier. Incubation mixtures containing no inhibitor were used simultaneously, as controls, in all experiments on inhibition. Percentage inhibition was calculated as being equal to the difference between the amount of nitrogen precipitated in the absence and presence of inhibitor, divided by that in the absence of inhibitor, and multiplied by 100.

## RESULTS

*Quantitative precipitation studies.* — The quantitative precipitation curves of concanavalin A with the levans from *A. levanicum*, *B. megatherium*, and *B. subtilis*, obtained by plotting μg of nitrogen found in the precipitate (by ninhydrin analysis) against mg of levan added, are shown in Figs. 1, 2, and 3, respectively. The carbohydrate contents of the concanavalin A–levan precipitates at each stage of the precipitation curves are also shown.

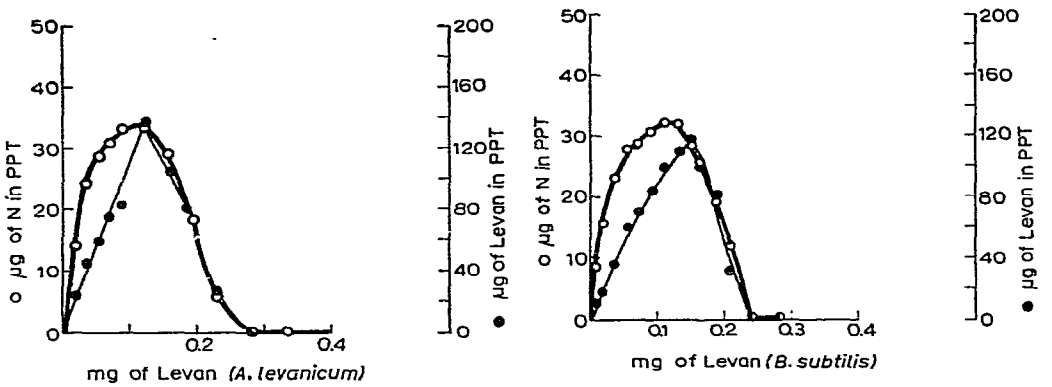



Fig. 1. Quantitative precipitation curve of *A. levanicum* levan with concanavalin A (48 μg of nitrogen). The total amount of levan precipitated is also shown.

Fig. 2. Quantitative precipitation curve of a levan from *B. subtilis* with concanavalin A (42 μg of nitrogen). The total amount of levan precipitated is also shown.

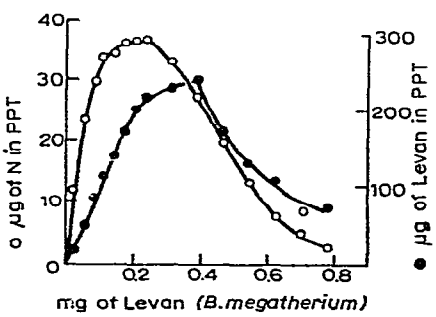



Fig. 3. Quantitative precipitation curve of a levan from *B. megatherium* with concanavalin A (49 μg of nitrogen). The amount of carbohydrate precipitated is also shown.

In contrast to dextran[8] B-1355-S and a variety of α-D-mannans[19], which precipitate 95–98% of the total concanavalin A added, these levans precipitated, at the region of maximum precipitation, only about 70–78% of the total concanavalin A added. In the region of an excess of concanavalin up to the point of maximum precipitation, virtually all of the levan added was found to be in the precipitates.

TABLE I

INHIBITION OF CONCANAVALIN A–POLYSACCHARIDE PRECIPITATION BY MONO- AND OLIGO-SACCHARIDES AND SOME OF THEIR DERIVATIVES

| *Saccharide* | *μMoles giving 50% inhibition in the dextran–concanavalin A system*[a] | *μMoles giving 50% inhibition in the levan–concanavalin A system*[b] |
|---|---|---|
| Methyl α-D-mannopyranoside | 0.6 | 0.087 |
| Methyl β-D-fructopyranoside | 0.85 | 0.12 |
| Methyl α-D-glucopyranoside | 2.5 | 0.36 |
| Methyl α-L-sorbopyranoside | 3.1 | |
| D-Mannose | 4.4 | |
| Methyl β-D-fructofuranoside | 5.7 | 0.94 |
| D-Fructose | 9.3 | 1.5 |
| Methyl α-D-fructofuranoside | 16 | 2.3 |
| D-Glucose | 21 | 3.5 |
| Methyl α-D-fructopyranoside | 107 (40%)[c] | |
| D-Allose | 97 (4%) | |
| D-Galactose | 104 (8%) | |
| Melezitose | 0.71 | |
| Leucrose | 0.85 | |
| Inulotriose | 4.6 | |
| Nystose | 4.9 | |
| Turanose | 5.6 | |
| Levanbiose | 6.9 | |
| 1-Kestose | 7.8 | |
| Inulobiose | 9.6 | |
| Di-D-fructose 1,2′:2,1′-dianhydride | 13.5 | |
| Sucrose | 23 | 4.0 |
| Planteobiose | 35 (25%) | |
| Planteose | 34 (9%) | |
| Raffinose | 100 (23%) | |
| Lactulose | 100 (4%) | |

[a]Each tube contained concanavalin A (300 μg), dextran NRRL-B-1355-S (200 μg), and inhibitor as noted, in a total volume of 1.0 ml. [b]Each tube contained concanavalin A (300 μg), *A. levanicum* levan (100 μg), and inhibitor as noted, in a total volume of 1.0 ml. [c]Numbers in parentheses refer to percentage inhibition given by the μmoles of saccharide noted.

Fig. 4 presents the curve for precipitation of concanavalin A and the D-gluco-D-fructan from the Hawaiian Ti plant. The interaction is relatively weak, as, of the 46 μg, only about 19 μg (41%) of concanavalin A nitrogen was precipitated at the point of maximum precipitation.

*Hapten-inhibition studies.* — Unless specified otherwise, hapten-inhibition experiments were conducted with the concanavalin A–dextran (B-1355-S) system[10].

The inhibition caused by several monosaccharides and their derivatives is shown in Table I. As in previous studies, D-mannose was found to be a more potent inhibitor than D-fructose, which, in turn, was superior to D-glucose; methyl α-D-glucopyranoside was approximately 8.4 times as potent as D-glucose.

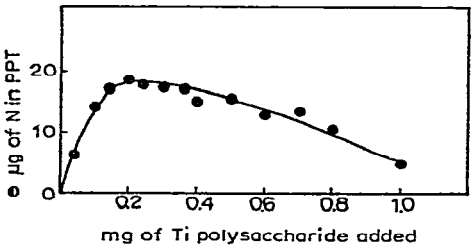



Fig. 4. Quantitative precipitation curve of the D-gluco-D-fructan from the Hawaiian Ti plant, with concanavalin A (46 μg of nitrogen).

Of the four methyl glycosides of D-fructose, methyl α-D-fructopyranoside was a relatively poor inhibitor (40% at 107 μmoles) compared with the three other methyl fructosides, the degree of potency decreasing in the order: methyl β-D-fructopyranoside > methyl β-D-fructofuranoside > methyl α-D-fructofuranoside. Methyl α-L-sorbopyranoside (3.1 μmoles for 50% inhibition) was a less potent inhibitor than methyl β-D-fructopyranoside.

A large number of anhydropentitols and anhydrohexitols were also investigated for their capacity to inhibit the concanavalin A–dextran system; these are listed in Table II. Of the anhydroalditols containing a tetrahydrofuran ring, only those containing the 1,4-anhydro-D-arabinitol ring-system were found to be inhibitors of

TABLE II

INHIBITION OF PRECIPITATION BY ANHYDRO ALDITOLS

| *Sugar* | *μMoles* | *Inhibition, %* |
|---|---|---|
| 1,4-Anhydro-DL-allitol | 57 | 2.2 |
| | 105 | 7.1 |
| 1,4-Anhydro-D-altritol | 56 | 5.2 |
| 1,4-Anhydro-D-arabinitol | 100 | 44 |
| 1,4-Anhydro-D-glucitol | 50 | 3.5 |
| 1,5-Anhydro-D-glucitol | 14 | 50 |
| 2,5-Anhydro-D-glucitol | 19 | 70 |
| 2,5-Anhydro-L-iditol | 55 | 1.8 |
| 1,4-Anhydro-D-lyxitol | 52 | 0 |
| 1,4-Anhydro-D-mannitol | 49 | 4.8 |
| 1,5-Anhydro-D-mannitol | 4.4 | 50 |
| 2,5-Anhydro-D-mannitol | 19 | 50 |
| 1,4-Anhydro-DL-ribitol | 52 | 0 |
| | 100 | 4 |
| 1,4-Anhydro-DL-xylitol | 47 | 3.8 |
| | 103 | 6.1 |

concanavalin A–polysaccharide precipitation. Thus, among the 1,4-anhydropentitols, only 1,4-anhydro-D-arabinitol was an inhibitor of any consequence.

Both 2,5-anhydro-D-mannitol (19 μmoles for 50% inhibition) and 2,5-anhydro-D-glucitol (70 μmoles for 50% inhibition) possess the requisite 1,4-anhydro-D-arabinitol ring-system, and hence behave as inhibitors. 1,5-Anhydro-D-mannitol and 1,5-anhydro-D-glucitol are considerably better inhibitors than their corresponding 2,5-anhydro derivatives. Structural correlations of all of these anhydroalditols, together with a rationale for their reactivity in the concanavalin A system, are presented in the Discussion.

Inhibition analysis of many of the D-pentofuranosides, together with that for several of the corresponding pyranosides, is presented in Table III, from which is evident that only methyl α- and β-D-arabinofuranoside bind to the active sites of

TABLE III

INHIBITION OF PRECIPITATION BY PENTOSES AND METHYL PENTOSIDES

| *Sugar* | *μMoles* | *Inhibition, %* |
|---|---|---|
| D-Arabinose | 110 | 24 |
| L-Arabinose | 96 | 6 |
| Methyl α-D-arabinofuranoside | 51 | 50 |
| Methyl β-D-arabinofuranoside | 60 | 50 |
| Methyl β-L-arabinopyranoside | 52 | 0.2 |
| Methyl α-D-lyxofuranoside | 60 | 4.3 |
| Methyl β-D-ribofuranoside | 54 | 0.5 |
| Methyl α-D-xylofuranoside | 52 | 0.2 |
| Methyl α-D-xylopyranoside | 64 | 4.6 |
| Methyl β-D-xylopyranoside | 58 | 3.1 |
| D-Ribose | 107 | 8 |
| D-Xylose | 106 | 5 |

the protein molecule. Furthermore, of the four D-aldopentoses, only D-arabinose (but not L-arabinose) inhibited the concanavalin A–dextran system. It may be noted that D-ribose, reported by Doyle *et al.*[20] to be somewhat active in the concanavalin A system, is without activity at a level of 107 μmoles.

Several oligosaccharides containing D-fructofuranosyl residues were also tested for their capacity to inhibit concanavalin A–polysaccharide interaction (see Table I). In the series of sucrose, 1-kestose [β-D-Fru*f*-(2→1)-β-D-Fru*f*-(2↔1)-α-D-G*p*], nystose [β-D-Fru*f*-(2→1)-β-D-Fru*f*-(2→1)-β-D-Fru*f*-(2↔1)-α-D-G*p*], the percentage inhibition increased in that order. Levanbiose (6.9 μmoles for 50% inhibition) is a slightly better inhibitor than inulobiose (9.6 μmoles for 50% inhibition), and inulotriose (4.6 μmoles for 50% inhibition) is superior to inulobiose. A difructosan composed of two β-D-fructofuranosyl residues united by 1,2′:2,1′-anhydride linkages gave 50% inhibition at a level of 13.5 μmoles. Leucrose [α-D-G*p*-(1→5)-D-Fru*p*; 0.85 μmole for 50% inhibition] and turanose [α-D-G*p*-(1→3)-D-Fru] (5.6 μmoles for 50% inhibition) were found to be better inhibitors than sucrose (23 μmoles for 50% inhibition).

Melezitose [α-D-Glc*p*-(1→3)-β-D-Fru*f*-(2↔1)-α-D-Glc*p*; 0.71 μmole for 50% inhibition] is almost as effective an inhibitor as methyl α-D-mannopyranoside (0.64 μmole for 50% inhibition); the latter is one of the most potent inhibitors of concanavalin A–polysaccharide precipitation thus far examined. With the exception of planteobiose [α-D-Gal*p*-(1→6)-D-Fru*f*; 35 μmoles for 25% inhibition], the remaining oligosaccharides containing D-fructofuranosyl moieties (raffinose, gentianose, planteose, and lactulose) were essentially ineffective as inhibitors of precipitation.

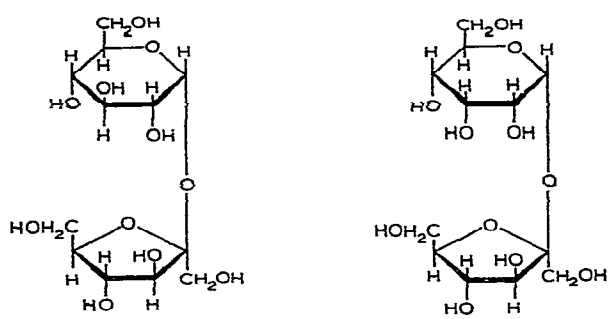
Several methyl ethers of D-fructose were examined for their inhibitory activity in the concanavalin A–polysaccharide reaction. It was found that modification of the 3- or 4-hydroxyl groups of D-fructose gave compounds that still produced significant inhibition of precipitation, 4-*O*-methyl-D-fructose (19 μmoles for 93% inhibition) being a more potent inhibitor than 3-*O*-methyl-D-fructose (19 μmoles for 30% inhibition) (see Table IV). The di-, tri-, and tetra-methyl ethers of D-fructose that were tested were found to be noninhibitory.

TABLE IV

INHIBITION OF PRECIPITATION BY METHYL ETHERS OF D-FRUCTOSE

| *Ether of* D-*fructose* | *μMoles* | *Inhibition, %* |
|---|---|---|
| 3-Methyl | 19 | 30 |
| 4-Methyl | 19 | 93 |
| 3,4-Dimethyl | 87 | 0 |
| 1,3,4-Trimethyl | 20 | 2 |
| 1,3,4,6-Tetramethyl | 32 | 7 |

Inhibition data obtained with sucrose and allosucrose [α-D-All*p*-(1↔2)-β-D-Fru*f*] demonstrated that, in sucrose, it is the D-glucosyl moiety that is principally involved in binding, despite the presence in the D-fructosyl moiety of the determinants necessary for interaction with concanavalin A. The difference in structure between sucrose and allosucrose lies only in the disposition of the 3-hydroxyl group of the hexopyranosyl moiety (see Fig. 5). At a level of 52 μmoles, allosucrose was a noninhibitor, whereas



Sucrose

23 μmoles; 50% inhibition

Allosucrose

52 μmoles; 0% inhibition

Fig. 5. Inhibition of precipitation by sucrose and allosucrose.

at 23 μmoles, sucrose gave 50% inhibition. It should be noted that D-allose is a noninhibitor of precipitation (4% at 97 μmoles).

Inhibition studies were conducted with the concanavalin A–levan (*A. levanicum*) system, and it was found that the relative order of activity of the different inhibitors examined paralleled exactly that shown for the concanavalin A–dextran (B-1355-S) system[10]; however, smaller proportions of the various sugars were required for the same percentage inhibition (Table I).

*Chromatography on cross-linked, levan gel.* — When a solution of protein from jack-bean meal, precipitated between 0.3 and 0.8 saturation with respect to $(NH_4)_2SO_4$, was passed through a small column of cross-linked, levan gel, a chromatographic pattern similar to that obtained with the Sephadexes was obtained[14]. All of the proteins unreactive with the levan matrix appeared with the void volume (peak No. 1). On addition of a solution of D-glucose (0.1M) to the column, the active protein was eluted (peak No. 2). About 60% of the total protein applied to the column was recovered in the first peak. The remaining protein that was displaced by D-glucose was active, the activity being determined by quantitative precipitation[8] with dextran B-1355-S after extensive dialysis to remove D-glucose. Peak No. 1 gave no precipitate with dextran B-1355-S.

D-Galactose, D-xylose, melibiose, cellobiose, and lactose did not displace the protein from the column, whereas either D-fructose or D-glucose, at a concentration of 100 mM, did. When a 10 mM solution of D-glucose was employed as eluant, the adsorbed protein was incompletely displaced.

Concanavalin A isolated by the Sephadex-adsorption method was specifically bound to cross-linked, levan gel, and was displaced as a single peak by D-glucose. No protein was eluted with the void volume of the column.

## DISCUSSION

Bacterial levans containing multiple, terminal β-D-fructofuranosyl residues have been shown herein to be precipitants of concanavalin A[4-6,21], maximum precipitations occurring at pH 6.0–7.2, which is the same pH range as observed with dextrans[8] and D-mannans[19]. Concanavalin A–levan (*A. levanicum*) precipitates, however, are more soluble (3.5 μg of N/ml)[22] compared with concanavalin A–dextran (1.5 μg of N/ml)[8] or concanavalin A–D-mannan (1.0 μg of N/ml)[19] precipitates. As with results obtained with other polysaccharides, precipitation curves of D-fructans with concanavalin A demonstrated, as in the case of antibody–antigen precipitation, the presence of three zones of precipitation; these are a zone of (*1*) concanavalin A excess, (*2*) "equivalence", and (*3*) levan excess. In the region of excess of concanavalin A, all of the levan added is completely precipitated. However, in the region of "equivalence" (or maximum precipitation of protein), incomplete precipitation of both the protein and the polysaccharide was observed. With *A. levanicum* levan as the precipitant, for example, only 72% of the total protein-nitrogen added was found in the precipitate, compared with a value of 95–98% for dextrans and D-mannans. The

levans from *B. subtilis* and *B. megatherium* precipitated only 74 and 78%, respectively, of the total concanavalin A at the region of maximum precipitation. This result is believed to be attributable to the low reactivity of this type of polysaccharide and to the greater solubility of concanavalin A–levan precipitates. This conclusion will become more evident from an evaluation of the inhibition experiments described later.

As an example of the interaction of a plant D-fructan with concanavalin A, we investigated the reaction with the D-gluco-D-fructan from the Hawaiian Ti plant. This polysaccharide was shown to possess, on the average, four terminal β-D-fructofuranosyl groups for every 14 hexose residues[23]. Agar-gel diffusion studies on the interaction of concanavalin A with this D-gluco-D-fructan, as well as with the several other levans investigated in the present work, have been published[4].

Studies in our laboratory have indicated that the combining sites of concanavalin A are complementary to the α-D-mannopyranosyl moiety, there being definite loci on the protein molecule for the hydroxyl groups on C-2, C-3, C-4, and C-6 of the pyranose ring and the α-D-glycosidic oxygen atom[24]. The α-D-glucopyranosyl moiety differs from the α-D-mannopyranosyl residue with respect only to the disposition of the 2-hydroxyl group, which, in the former species, is a destabilizing factor. For this reason, it is not surprising that 1,5-anhydro-D-mannitol (which contains four binding loci) is a good inhibitor of concanavalin A–polysaccharide interaction, being superior to 1,5-anhydro-D-glucitol (which contains only three binding loci).

For the purposes of the subsequent discussion, a generalisation may now be formulated: the minimum structural feature required for the binding of furanose sugars or their derivatives to concanavalin A is the 1,4-anhydro-D-arabinitol moiety. Thus, of the several 1,4-anhydropentitols and 2,5-anhydrohexitols (see Table II) that were examined as inhibitors of the concanavalin A–polysaccharide system, only 1,4-anhydro-D-arabinitol, 2,5-anhydro-D-glucitol, and 2,5-anhydro-D-mannitol were active. Similarly, only the methyl α- and β-D-arabinofuranosides (51 and 60 μmoles, respectively, for 50% inhibition), out of all the methyl pentofuranosides tested (see Table III), were inhibitors, these D-arabinofuranosides being superior to 1,4-anhydro-D-arabinitol (100 μmoles for 44% inhibition) and D-arabinose (110 μmoles for 20% inhibition). The enantiomorphic L-arabinose was, as expected, a noninhibitor.

Of the four methyl glycosides of D-fructose, three were found to be significant inhibitors of concanavalin A–polysaccharide interaction. Methyl β-D-fructopyranoside was the most potent inhibitor of precipitation (0.85 μmole for 50% inhibition), the inhibition being close to that of methyl α-D-mannopyranoside (0.60 μmole for 50% inhibition). Examination of the structure of this D-fructoside (see Fig. 6) reveals that it is configurationally related to 1,5-anhydro-D-mannitol. Methyl α-L-sorbopyranoside, which is configurationally similar to 1,5-anhydro-D-glucitol, is a poorer inhibitor than methyl β-D-fructopyranoside. Methyl α-D-fructopyranoside is essentially ineffective as an inhibitor.

The binding of furanoid sugars to concanavalin A may be rationalized on the basis of common configurational features with sugars possessing the pyranoid ring.

Thus, the disposition of the hydroxyl groups on C-3, C-4, and C-6 of the D-fructofuranosyl residue is similar to the orientation of the hydroxyl groups on C-3, C-4, and C-6 of the D-glucopyranosyl (or D-mannopyranosyl) residue. The greater potency of methyl β-D-fructofuranoside (16 μmoles for 50% inhibition) and 2,5-anhydro-D-mannitol (19 μmoles for 50% inhibition) over that of 2,5-anhydro-D-glucitol

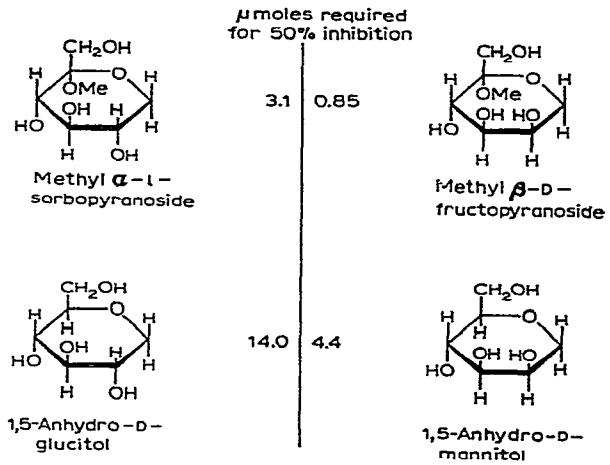



Fig. 6. Structural correlation and inhibition potency of two methyl hexulosides and two anhydroalditols.

(70 μmoles for 50% inhibition) suggests a further possibility, namely, binding at the 1-, 3-, and 4-hydroxyl groups of methyl β-D-fructofuranoside and 2,5-anhydro-D-mannitol. The 3-fold increase in inhibiting power of methyl β-D-fructofuranoside relative to that of 2,5-anhydro-D-mannitol, and the 4-fold increase in inhibiting power of methyl α-D-fructofuranoside relative to that of 2,5-anhydro-D-glucitol, indicate that the aglycon of these methyl D-fructofuranosides may in some way contribute to the binding; this, however, requires further investigation.

The binding of D-arabinose, D-fructose, and L-sorbose to the active sites of concanavalin A may be explained by the presence of the appropriate ring forms of these sugars in aqueous solution[25].

Inspection of Dreiding models of the D-glucopyranosyl and D-fructofuranosyl ring-systems reveals remarkable similarities in the angular dispositions of the critical hydroxyl groups of the two ring-systems (C. T. Bishop, personal communication). For example, if the hydroxyl group of the hydroxymethyl group is placed at its maximum distance from the neighboring, secondary hydroxyl group, with the line of vision down the C-5–H and C-3–H bonds, it is found that the angle subtended by O-6–O-4–O-3 in the D-glucopyranose ring is 121°. With the furanose ring in the $T_2^3$ conformation[26], the O-5–O-3–O-2 angle is exactly the same.

It is noteworthy that the β-D-fructofuranosyl ring, the residue present at the chain ends of levans, possesses only *three* definitive binding-sites with respect to the combining sites of the concanavalin A molecule, namely, the hydroxyl groups at

C-3, C-4, and C-6 (or C-1, C-3, and C-4). This situation contrasts with the *five* sites of attachment identified for the α-D-mannopyranosyl residue and the *four* sites identified for the α-D-glucopyranosyl moiety. Thus, it is not surprising that the interaction of levans with concanavalin A is of a lower order than that for α-D-mannans and α-D-glucans. It is, of course, essential that the frequency of branching and the number of glycosyl residues in the outer chains of a polysaccharide molecule be also considered in assessing the reactivity of carbohydrate polymers with concanavalin A.

Additional evidence showing that binding to the D-fructofuranose ring can occur at the 3-, 4-, and 6-hydroxyl groups is forthcoming from a consideration of the inhibition of precipitation observed with di-D-fructose 1,2′:2,1′-dianhydride (50% at 13.5 μmoles). This compound contains two D-fructofuranose ring systems[23,27,28] joined by 1,2′:2,1′ anhydride linkages; thus, the only possibility by which binding to concanavalin A can occur is at the hydroxyl groups at C-3, C-4, and C-6, or at C-3′, C-4′, and C-6′ of the sugar, or both. Since the hydroxyl groups at C-1 and C-2 of both of the D-fructofuranosyl residues are involved in anhydride ring-formation, simultaneous binding at positions C-1, C-3, and C-4 cannot occur.

Inhibition obtained with planteobiose indicates that binding of the D-fructofuranosyl ring to concanavalin A can also occur at the 1-, 3-, and 4-hydroxyl groups. Since D-galactose (8% inhibition at 104 μmoles) and melibiose [α-D-Gal*p*-(1→6)-D-G*p* (4% inhibition at 70 μmoles] were without effect on the precipitation, it follows that any inhibition observed with planteobiose must result from combination with the D-fructofuranosyl residue. In a solution of planteobiose, both the α- and β-D-furanose forms are likely to be present. Of these, only the β-D anomer would be expected to exhibit affinity for the receptor sites of concanavalin A. The low inhibition observed with planteobiose (25% at 35 μmoles) suggests that only a small proportion of the β-D anomer exists in an aqueous solution of this disaccharide. Quite interestingly, the trisaccharide planteose [α-D-Gal*p*-(1→6)-β-D-Fru*f*-(2↔1)-α-D-G*p*] does not produce any significant inhibition of precipitation at 35 μmoles, although the 3-, 4-, and 6-hydroxyl groups of the terminal α-D-glucopyranosyl residue, as judged by the Haworth structure, are free to interact. Inspection of a molecular model of this compound, however, suggested that the D-galactose residue may sterically block the essential hydroxyl groups on the nonreducing α-D-glucopyranosyl group.

A particularly interesting inhibitor of concanavalin A-polysaccharide precipitation is the disaccharide sucrose. In this sugar, both the α-D-glucopyranosyl group and β-D-fructofuranosyl group possess the necessary determinants for binding to the receptor sites of concanavalin A. Were both glycosyl groups actually involved in binding to the protein, sucrose would be expected to be a very potent inhibitor, assuming that both groups could occupy the active sites of concanavalin A without significant steric hindrance by the other. However, the affinity of sucrose for concanavalin A is of only the same order of magnitude as that of D-glucose (21 μmoles for 50% inhibition). Inhibition data obtained with "allosucrose" (52 μmoles for 0% inhibition), in which the D-glucosyl group of sucrose has been transformed into

a D-allosyl group, showed a greatly diminished inhibition, demonstrating that it is principally the α-D-glucopyranosyl group of sucrose that binds to concanavalin A.

Inhibition of concanavalin A–polysaccharide interaction by additional oligosaccharides containing D-fructofuranosyl residues indicated that the binding of sugars to concanavalin A may increase as additional D-fructofuranosyl groups are glycosidically linked to sucrose. Thus, inhibition of precipitation increases in the series of sucrose, 1-kestose, and nystose. Apparently, the serial addition of D-fructofuranosyl groups increases the number of potential binding-sites. As the series is ascended, the limiting factor may, perhaps, be the stabilization of the linear molecule by intramolecular hydrogen-bonds. It is still unknown whether a limit in inhibiting power may be attained. This is precisely the phenomenon that was observed when a homologous series of α-(1→2)-linked D-mannopyranosyl residues was examined as inhibitors of the concanavalin A–polysaccharide system[19].

It is interesting that inulin, which consists of a linear chain of about 35 (2→1)-linked β-D-fructofuranosyl residues terminated by a sucrose moiety, is not a precipitant of concanavalin A, but is an inhibitor of precipitation. The inhibiting power of inulin is of similar magnitude to that of methyl α-D-glucopyranoside, which is 2.5 times that of nystose.

Of the methyl ethers of D-fructose examined, only 3-*O*-methyl- and 4-*O*-methyl-D-fructose inhibited the concanavalin-A reaction significantly. Inhibition studies must be conducted on all of the possible methyl ethers of D-fructose before any further conclusions can be drawn.

The hapten-inhibition experiments described were conducted with the concanavalin A–dextran system. When these studies were repeated with levan as the precipitating polysaccharide (see Table I), the relative order of activity of the inhibitors tested was found to be precisely the same as that for the concanavalin A–dextran system, except that relatively smaller proportions of the individual saccharides were required. This result suggests that the binding of levan to concanavalin A is substantially weaker than that of dextran, and, hence, more readily inhibited. In both systems, methyl β-D-fructofuranoside (corresponding to the sugar residue at the chain end of levan molecules) is a poorer inhibitor than methyl α-D-glucopyranoside (corresponding to the sugar residue at the chain ends of α-D-glucans). The results of similar studies conducted with the concanavalin A–glycogen[9] and concanavalin A–D-mannan[19] systems parallel the inhibition pattern described for the concanavalin A–dextran system. These data provide strong evidence that the interaction of concanavalin A with polysaccharides occurs at the same receptor sites on the protein molecule. The fact that concanavalin A that had been isolated on Sephadex can be specifically bound to cross-linked, levan gel, and then subsequently be eluted with the same sugars employed to elute the protein from Sephadex supports this view.

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