

Protein-Carbohydrate Interaction

V. Further Inhibition Studies Directed toward Defining the Stereochemical Requirements of the Reactive Sites of Concanavalin A

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Inhibition studies on the concanavalin A-dextran interaction have been extended using as inhibitors the members of the maltose-, isomaltose-, and methyl α -isomaltoside series, amino and *N*-acetyl amino sugars, and a number of oligosaccharides containing more than one linkage type. The interaction of the plant protein at the terminal chain ends of the α -linked glucans and mannans has been essentially confirmed, and the different characteristic inhibition activities of the linkage types is discussed in terms of steric effects. A possible application of the inhibition technique towards structural analysis of carbohydrate molecules is suggested by the authors.

A previous paper in this series (1) elaborated in some detail the configurational features of low molecular weight oligosaccharides which are essential for binding to the reactive sites of the concanavalin A protein molecule. On the basis of inhibition studies it was suggested that the specificity of the protein is directed primarily toward the C-3, C-4, and C-6 hydroxyl groups of the *D*-mannopyranosyl and *D*-glucopyranosyl ring forms which possess the α -configuration at C-1.

In attempting to account, on a molecular level, for the capacity of concanavalin A to interact to form a precipitate with a restricted group of branched polysaccharides [glycogens, dextrans, amylopectin, yeast mannans, and more recently, bacterial levans (2)], it was suggested that the protein com-

bins with the terminal nonreducing units of the polysaccharide chains. In this communication further confirmation of this hypothesis is provided by a more detailed examination of the inhibition of the dextran-concanavalin A interaction, using the members of several homologous series, a number of oligosaccharides containing more than one type of linkage, and some amino sugars as inhibitors.

EXPERIMENTAL PROCEDURE

The preparation of concanavalin A and the assay procedure used for inhibition studies have already been described (1). Dextran NRRL B-1355-S was employed as the precipitating polysaccharide.

Many of the saccharides used in this study were gifts (see acknowledgments). All were tested for purity by paper chromatography. Impure preparations were purified by preparative chromatography on Whatman 3MM or S and S Green Label filter paper.

Sugar solutions of known concentration were prepared by weighing in the case of crystalline compounds, the crystals having been previously dried to constant weight. Noncrystalline substances were determined by the phenol-sulfuric

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acid procedure (3). The hetero-oligosaccharides were also determined by this method using synthetic standards containing a suitable ratio of the component sugars.

Preparation of nigerosyl erythritol. To an aqueous solution of isolichenin (0.513 gm 25 ml) was added a solution of sodium metaperiodate (0.23 m, 10 ml) and the volume adjusted to 50 ml. The oxidation was allowed to proceed at 25° in the dark. After 3 days 0.35 mole of oxidant per glucosyl residue had been consumed. Solid BaCO₃ was added to neutrality and the solids were centrifuged. The supernatant solution was poured into 10 ml of water containing sodium borohydride (200 mg). After 24 hours at room temperature an additional portion of NaBH₄ (100 mg) was added. On the following day the solution was made just acid to litmus with acetic acid and poured with stirring into 95% ethanol (400 ml). The precipitate was centrifuged, washed several times with 95% ethanol and successively with absolute ethanol, diethyl ether, and light petroleum ether, and dried *in vacuo* at 25°. Yield: 360 mg of white powder.

The periodate-oxidized and reduced isolichenin (300 mg) was dissolved in 0.2 N sulfuric acid (50 ml) and placed in a water bath at 60° for 30 minutes. The solution was neutralized (BaCO₃), filtered, and concentrated. Paper chromatography, with ethyl acetate:pyridine:water (10:4:3, v/v) as the development solvent and silver nitrate-sodium hydroxide spray reagent, revealed that the main components of the hydrolyzate were nigerosyl erythritol (I) and glucosyl erythritol, with traces of glucose and erythritol. Nigerosyl erythritol was isolated by preparative paper chromatography as a syrup (99 mg) and had $[\alpha]_D^{25} + 184^\circ$ in water (*c*, 0.66), identical with an authentic specimen isolated by Goldstein and Whelan (4). A recent communication (5) also reports the isolation of nigerosyl erythritol from isolichenin.

A portion of I (52.8 mg) was oxidized in 0.023 M sodium periodate (10 ml) at 25° in the dark. After 48 hours, 3.3 M proportions of periodate had been consumed with the production of 0.9 mole of formic acid and 1.1 moles of formaldehyde (theoretical ratio: 3:1:1). The reaction mixture was neutralized (BaCO₃) and centrifuged, and the supernatant solution was treated with NaBH₄ (20 mg). The reduced material was deionized by passing the solution successively through Amberlite IR 120 H and IR 45 A exchange resins. Borate was removed by several evaporations in the presence of methanol and the product was recovered as a syrup (II).

A portion of syrup II was dissolved in 0.2 N sulfuric acid and allowed to stand at 20° for 16 hours. The solution was neutralized (BaCO₃), filtered, and concentrated to yield a syrup which

was shown by paper chromatography to contain glucosyl glycerol (III) and glycerol. Glycerol was characterized as the tri-*p*-nitrobenzoate derivative (m.p. and mixed m.p. 188–191°); the glucosyl glycerol (2-*O*- α -D-glucopyranosyl-glycerol) had $[\alpha]_D^{25} + 125^\circ$ in water (*c*, 1.3); Charlson *et al.* (6) report $[\alpha]_D^{27} + 121^\circ$ in water.

RESULTS

As in the former study (1), we have arbitrarily classed as noninhibitors those substances of which 20 μ moles gave less than 10% inhibition of the concanavalin A reaction. Table I lists all the substances tested in this study. Three homologous series have been investigated: the maltodextrins (maltose to maltodecaose inclusively), the iso-maltodextrins (isomaltose, isomaltotriose, -tetraose, and -heptaose), and the methyl α -isomaltosides (methyl isomaltoside to methyl α -isomaltooctaoside inclusively).

On a molar basis, the members of the maltodextrin series exhibited the same inhibition activity and fell on a single inhibition curve (Fig. 1a). Similarly, the inhibition values for the isomaltodextrins, and the corresponding methyl α -glycosides which were tested, also can be fitted to a single curve (Fig. 1b). The results of inhibition studies on a number of oligosaccharides containing more than one type of glycosidic linkage are given in Fig. 2. Trisaccharide B (Panose), tetrasaccharide C (7) (see Fig. 3) and maltosan (4-*O*- α -D-glucopyranosyl-1,6-anhydro- β -D-glucopyranose) all exhibited an inhibition activity equal to that of the isomaltose series (1.1 μ moles gave 50% inhibition). The inhibition activity observed for both methyl β -maltoside and trisaccharide E (Isonanose) (2.0 μ moles for 50% inhibition) closely approached that of the maltose series. Trisaccharide F, which differs from E in that the (1 \rightarrow 6)-glucosidic linkage has the β rather than α -configuration, is an inhibitor somewhat better than maltose, but trisaccharide G, in which the (1 \rightarrow 4) bond is also in the β -configuration, is a noninhibitor.

The two branched trisaccharides³ J (8) and K also differ only in that K has a β (1 \rightarrow 6)-linkage; however, in this case, the presence

³ Saccharides in which at least two sugar residues are glycosidically linked to a third sugar residue.

TABLE I
SUBSTANCES TESTED FOR INHIBITION ACTIVITY

Inhibitors	Noninhibitors
Methyl β -D-mannopyranoside ^a	2-Amino-2-deoxy-D-glucose
Methyl 2-acetamido-2-deoxy- α -D-glucopyranoside	2-Amino-2-deoxy-D-mannose
Methyl 2-acetamido-2-deoxy- β -D-glucopyranoside ^b	Methyl 3-amino-3-deoxy- α -D-glucopyranoside
2-O- α -D-Glucopyranosyl-glycerol	6-Amino-6-deoxy-D-glucose
Mannosyl-glycerol	6-O-Acetyl-D-glucose
6-O- α -D-Mannopyranosyl-D-glucose	6-Deoxy-6-fluoro-D-glucose
O-(2-Acetamido-2-deoxy- α -D-glucopyranosyl)-(1 \rightarrow 3 or 4)-D-galactitol	4-O- β -D-Mannopyranosyl-D-mannose
O-(2-Acetamido-2-deoxy- α -D-glucopyranosyl)-(1 \rightarrow 6)-2-acetamido-2-deoxy-D-glucose	4-O- β -D-Glucopyranosyl-D-mannose
2-O- α -Nigerosyl-D-erythritol	O- β -D-Glucopyranosyl-(1 \rightarrow 4)- β -D-glucopyranosyl-(1 \rightarrow 6)-D-glucose
Methyl β -maltoside	O-(2-Acetamido-2-deoxy- β -D-glucopyranosyl)-(1 \rightarrow 4)-2-acetamido-2-deoxy-D-glucose
4-O- α -D-Glucopyranosyl-1,6-anhydro- β -D-glucopyranose	α -Schardinger dextrin
Maltose to maltodecaose, inclusively	β -Schardinger dextrin
Isomaltose	Trehalosamine
Isomaltotriose	Galactinol
Isomaltotetraose	N-Acetyl neuraminic acid
Isomaltoheptaose	
Methyl α -isomaltoside to methyl α -isomaltooctaside, inclusively	
Isopanose	
Panose	
Additional oligosaccharides in Fig. 3	

^a Relatively poor inhibitor.

^b Very poor inhibitor.

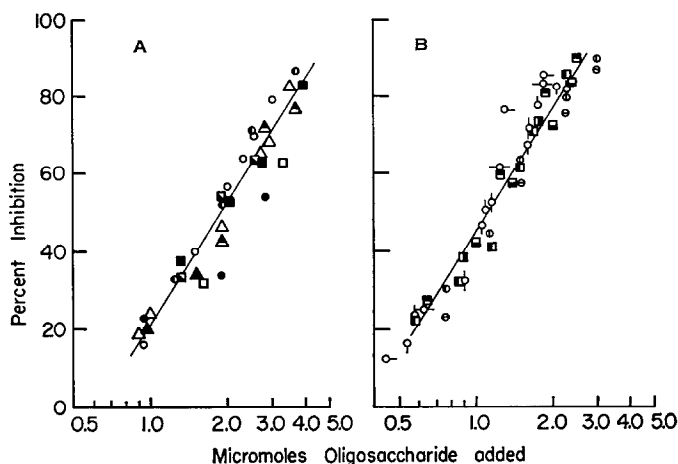


FIG. 1. (a) Inhibition of precipitation by the maltose series of oligosaccharides. \circ , Maltose; \bullet , maltotriose; \triangle , maltotetraose; \blacktriangle , maltopentaose; \square , maltohexaose; \blacksquare , maltoheptaose; \bullet , malto-octaose; \blacktriangle , maltononaose; \blacksquare , maltodecaose. (b) Inhibition of precipitation reaction by the isomaltose series of oligosaccharides. \blacksquare , Isomaltose; \square , isomaltotriose; \blacksquare , isomaltotetraose; \square , isomaltoheptaose; \circ , methyl- α -isomaltoside; \ominus , methyl α -isomaltotriose; \odot , methyl α -isomaltotetraose; \ominus , methyl α -isomaltopentaose; \odot , methyl α -isomaltohexaose; \odot , methyl α -isomaltoheptaose; \circ , methyl α -isomalto-octaose.

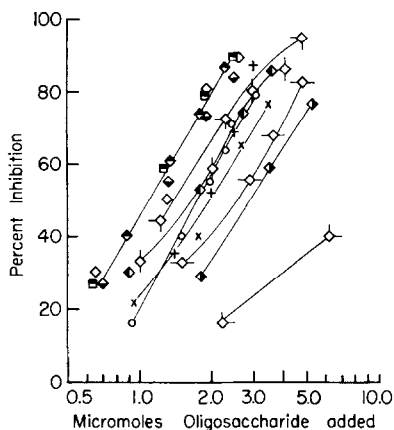


FIG. 2. Inhibition of precipitation by saccharides. \blacksquare , Isomaltose (A); \blacklozenge , panose (B); \blacklozenge , tetrasaccharide (C); \circ , maltose (D); $+$, isopanose (E); \blacklozenge , trisaccharide (F); \blacklozenge , tetrasaccharide (H); \blacklozenge , trisaccharide (J); \blacklozenge , trisaccharide (K); \blacklozenge , tetrasaccharide (L); \blacklozenge , pentasaccharide (M); \times , methyl β -maltoside; \blacklozenge , maltosan.

of the β -linkage resulted in K being a less effective inhibitor (9.0 moles for 50% inhibition) than trisaccharide J (2.3 μ moles for 50% inhibition). The branched pentasaccharide M (9) has a similar inhibiting activity to that of maltose.

The α - and β -Schardinger dextrans showed a complete lack of inhibition of the dextran-concanavalin A interaction. These molecules do not possess terminal nonreducing ends because they consist of α -D-(1 \rightarrow 4)-linked

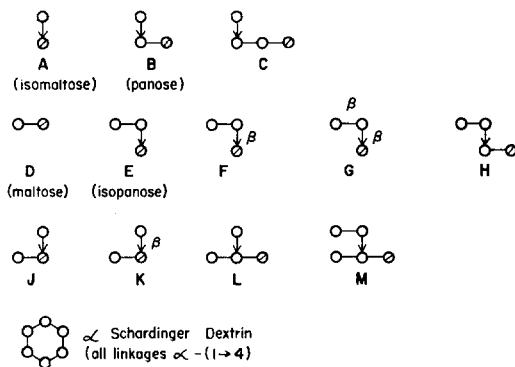


FIG. 3. Structure of some oligosaccharides tested for inhibition activity. \circ , Nonreducing glucose units; \bullet , reducing glucose units; (—), 1 \rightarrow 4 linkages; \downarrow , 1 \rightarrow 6 linkages; all α -linkages except where indicated.

glucosidic units in the form of six- or seven-membered rings, respectively.

The inhibition activity of nigerosyl erythritol (compound I, Fig. 4) is shown in Fig. 5 (5.0 μ moles for 50% inhibition). The product of periodate oxidation followed by reduction, compound II, failed to inhibit the dextran-concanavalin A interaction, whereas glucosyl glycerol (2-O- α -D-glucopyranosyl-glycerol (III), formed from II by mild acid hydrolysis (Fig. 4), exhibited an inhibition (1.2 μ moles for 50% inhibition) comparable to that of the isomaltose series. Two D-glucose derivatives (6-O-acetyl-D-glucose and 6-deoxy-6-fluoro-D-glucose) were found, as expected, to be noninhibitors, further confirming the requirement for an unmodified hydroxyl group at C-6.

Five amino sugars were tested (after neutralizing their solutions to pH 7.0) for their inhibition of the dextran-concanavalin A interaction. As expected, 6-amino-6-deoxy-D-glucose and methyl 3-amino-3-deoxy- α -D-

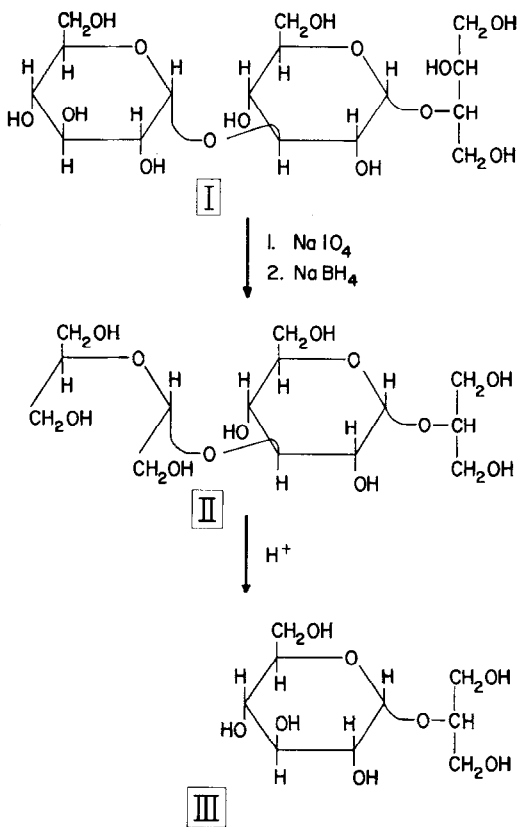


FIG. 4

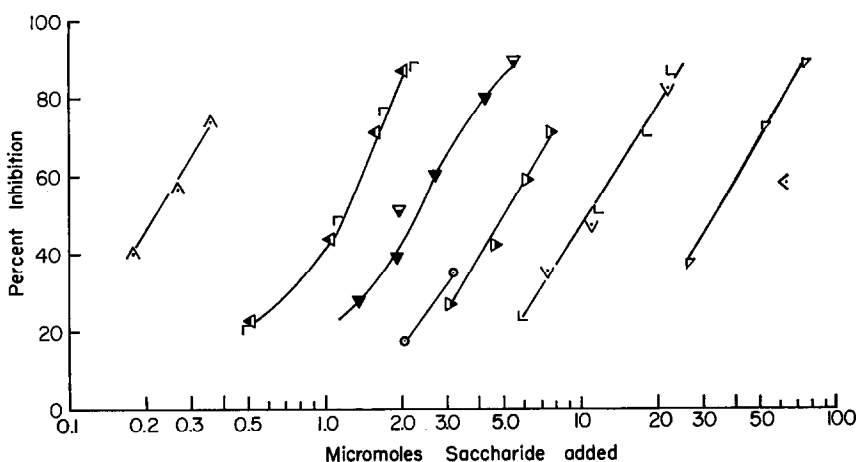


FIG. 5. Inhibition of precipitation by saccharides. ▼, Methyl 2-acetamido-2-deoxy- α -D-glucopyranoside; ◄, methyl 2-acetamido-2-deoxy- β -D-glucopyranoside; ▽, *O*-(2-acetamido-2-deoxy- α -D-glucopyranosyl)-(1 → 6)-2-acetamido-2-deoxy-D-glucose; ►, nigerosyl erythritol; ◄, 2-*O*- α -D-glucopyranosyl-glycerol; △, 6-*O*- α -D-mannopyranosyl-D-glucose; ∇, methyl β -D-mannopyranoside; ▢, methyl α -D-glucopyranoside; ▣, methyl β -D-glucopyranoside; L, D-glucose; ○, *O*-(2-acetamido-2-deoxy- α -D-glucopyranosyl)-(1 → 3 or 4)-D-galactitol.

glucopyranoside failed to inhibit, but surprisingly, 2-amino-2-deoxy-D-glucose, 2-amino-2-deoxy-D-mannose, and trehalosamine (α -D-glucopyranosyl-3-amino-3-deoxy- α -D-glucopyranoside) also failed to inhibit the interaction.

On a molar basis, the inhibition activities of the α - and β -methyl glycosides of 2-acetamido-2-deoxy-D-glucose were approximately 50% of the corresponding parent methyl α - and β -D-glucopyranosides. The disaccharide *O*-(2-acetamido-2-deoxy- α -D-glucopyranosyl)-(1 → 6)-2-acetamido-2-deoxy-D-glucose is somewhat less effective as an inhibitor (1.9 μ moles for 50% inhibition) than isomaltose (1.1 μ moles for 50% inhibition), whereas *N,N'*-diacetylchitobiose is a noninhibitor. A sample of *O*-(2-acetamido-2-deoxy- α -D-glucopyranosyl)-(1 → 3 or 4)-D-galactitol showed an inhibition of 35% for 3.2 μ moles, demonstrating the importance of the α -glycosidic linkage.

Of the mannose-containing sugars tested, it was again observed that the presence of the β -configuration at C-1 of the nonreducing sugar residue greatly reduced or completely abolished inhibition activity. Thus, methyl β -D-mannopyranoside, although more effec-

tive than the corresponding glucoside, is a relatively poor inhibitor and corresponds closely to the inhibition activity of glucose (11 μ moles for 50% inhibition), whereas two β -linked disaccharides, 4-*O*- β -D-mannopyranosyl-D-mannose (mannobiose) and 4-*O*- β -D-glucopyranosyl-D-mannose, can be classed as noninhibitors. On the other hand the α -linked disaccharide, 6-*O*- α -D-mannopyranosyl-D-glucose, was one of the most potent inhibitors tested (0.22 μ mole for 50% inhibition).

Galactinol and *N*-acetyl neuraminic acid were both found to be noninhibitors.

DISCUSSION

From earlier inhibition (1) studies it was anticipated that the D-glucose derivatives modified at C-3 and C-6 (6-*O*-acetyl-D-glucose, 6-deoxy-6-fluoro-D-glucose, methyl 3-amino-3-deoxy α -D-glucopyranoside, and 6-amino-6-deoxy-D-glucose) would not be inhibitory, but the finding that 2-amino-2-deoxy-D-glucose and trehalosamine also were inactive was unexpected inasmuch as 2-acetamido-2-deoxy-D-glucose previously had been shown to be an inhibitor equivalent in activity to D-glucose (1). The

occurrence of a positive charge on the free amino group (pK 7.8) (10) at pH 7.0 may account for the apparent failure of these sugars to interact with the concanavalin A protein. In this regard, 2-amino-2-deoxy-D-mannose was also a noninhibitor. *N*-Acetylation precludes the possibility of such a charge effect in the case of 2-amino-2-deoxy-D-glucose, and it was noted that the anomeric methyl glycosides of 2-acetamido-2-deoxy-D-glucose inhibited dextran-concanavalin A interaction, the α -anomer showing 50% inhibition for 2.3 μ moles and the β -anomer giving 50% inhibition for 60 μ moles; these values are approximately one half those found for the parent methyl α - and β -glucosides. Previously it had been shown that 2-acetamido-2-deoxy-D-mannose was a noninhibitor (1). These results indicate that although modification at the C-2 position of D-glucose is tolerated, a 50% reduction in inhibiting potency is noted when the hydroxyl group at C-2 of methyl α - and β -glucopyranoside is transformed into a 2-acetamido-2-deoxy group.

Two disaccharides and a disaccharide alcohol, all containing 2-acetamido-2-deoxy-D-glucose residues in the nonreducing positions, were examined. As expected, by virtue of its β -glycosidic linkage, *N,N'*-diacetyl chitobiose [*O*-(2-acetamido-2-deoxy- β -D-glucopyranosyl) - (1 \rightarrow 4) - 2 - acetamido - 2 - deoxy-D-glucose] was a noninhibitor. On the other hand, *O*-(2-acetamido-2-deoxy- α -D-glucopyranosyl) - (1 \rightarrow 6) - 2 - acetamido - 2 - deoxy-D-glucose, which contains an α -glycosidic bond, is a good inhibitor, being 60% that of isomaltose. Similarly *O*-(2-acetamido-2-deoxy- α -D-glucopyranosyl)-(1 \rightarrow 3 or 4)-D-galactitol proved to inhibit dextran-concanavalin A interaction. These observations suggest that glycoproteins which contain nonreducing terminal 2-acetamido-2-deoxy- α -D-glucopyranosyl units may also interact with concanavalin A.

The specific requirement for the α -configuration at C-1 of the interacting sugar residue is further confirmed by the observed lack of inhibition of the dextran-concanavalin A reaction by mannobiose (4-*O*- β -D-mannopyranosyl-D-mannose) and trisaccharide G (Fig. 3), and by the rather low inhibi-

tion activity of methyl β -D-mannopyranoside. In contrast are the very high activities of the α -linked compounds (Fig. 5), methyl α -D-mannopyranoside (1) and the disaccharide 6-*O*- α -D-mannopyranosyl-D-glucose. These data prompt the suggestion that glycoproteins containing terminal α -D-mannopyranosyl units should interact with concanavalin A.

The hypothesis that it is predominantly the terminal nonreducing α -D-glycopyranosyl residues of simple and complex α -glucans and α -mannans with which concanavalin A interacts (1, 11) received additional support from the fact that higher members of the three homologous series of saccharides tested have the same inhibition activities for the concanavalin A interaction as the corresponding disaccharide members. The identical inhibition values of panose and tetrasaccharide C (Fig. 2) with that of the isomaltose and methyl α -isomaltose series (1.1 μ moles for 50% inhibition) can be related to the observation that both of these oligosaccharides possess an isomaltose unit (6-*O*- α -D-glucopyranosyl-D-glucose) at their nonreducing ends. Similarly, the possession of a maltose residue (4-*O*- α -D-glucopyranosyl-D-glucose) at the terminal end of methyl β -maltoside and isopanose (trisaccharide E, Fig. 3) confers on these oligosaccharides an inhibition activity (2.0 μ moles for 50% inhibition) very close to that of the maltose series (1.9 μ moles for 50% inhibition). Although trisaccharide F (Fig. 3) and maltosan also contain a maltose-like structure, they exhibited inhibition activities (1.3 and 1.1 μ moles, respectively, for 50% inhibition) more characteristic of the isomaltose series. No explanation can be offered for the high inhibition of trisaccharide F, but it may be significant that in maltosan the C-6 hydroxyl group of the "reducing" glucosyl residue is involved in anhydro ring formation. It has been previously reported (1) that isomaltose, in which the C-6 hydroxyl of the reducing glucose residue is engaged in the glucosidic linkage, has a higher inhibition activity than any of the other α -linked glucose disaccharides tested. On the basis of these observations and of the similarity of the isomaltose inhibition activity to that of methyl α -D-

glucoside, it was suggested that the different apparent affinities of the disaccharides for the concanavalin A receptor sites could be explained by steric considerations, rather than by any real specificity difference for the linkage types. It is possible that the unexpectedly high inhibition activity observed for maltosan may also be explained by steric factors.

The branched trisaccharide J (Fig. 3), although possessing two terminal nonreducing α -D-glucopyranosyl residues, has an inhibiting activity surprisingly rather less than that of maltose. It seems probable that the greater part of the inhibition is a function of the constituent isomaltose unit, because in trisaccharide K, in which the 1 \rightarrow 6 bond is in the β -configuration, the inhibition is less by nearly four times. It is again possible to rationalize this relatively low inhibition activity of J in terms of a steric effect if one regards this trisaccharide either as an isomaltose derivative with a large substituent radical at C-4 of the reducing glucose unit, or as a maltose molecule carrying a large radical at C-6 of the reducing glucosyl residue. In pentasaccharide M (Fig. 3) one of the nonreducing ends may also be regarded as a maltose residue substituted in the reducing glucosyl moiety; the second nonreducing end may be considered as an unsubstituted maltose residue. Only the latter possibility would be expected to contribute significantly to the inhibition activity of the molecule as a whole, and this would seem to be borne out by the similarity of the observed inhibition (2.3 μ moles gave 50% inhibition) to that of maltose.

The failure of the α - and β -Schardinger dextrans to inhibit is consistent with the absence of any terminal glucose residues in their ring-like molecules. Possibly the best direct evidence for the interaction of concanavalin A with the saccharide chain-ends is provided by the inhibition studies carried out on nigerosyl erythritol and its oxidation and acid hydrolysis products. Nigerosyl erythritol (compound I, Fig. 3), having an unsubstituted terminal α -D-glucosidic residue, inhibited (50% for 5.0 μ moles) the dextran-concanavalin A precipitation reaction, whereas compound II, carrying a sub-

stituent residue at C-3 of the remaining intact α -D-glucosidic residue, failed completely to inhibit. Removal of this C-3 substituent, by mild acid hydrolysis, again presented an unsubstituted terminal α -D-glucosidic residue for interaction with concanavalin A, with the results that the product, 2-O- α -D-glucopyranosyl-glycerol (compound III), exhibited a high inhibition activity.

Although the possibility still exists that the interaction of the protein may extend in certain cases beyond the terminal chain end of a carbohydrate molecule (indeed this is indicated by the high inhibition activities observed for 3-O- α -D-glucopyranosyl-D-arabinose and for melezitose) (1), the data presented in this and the previous paper provide a fairly consistent conception of the stereochemical requirements of the interaction between concanavalin A and the terminal nonreducing chain-ends of the α -linked glucose- and mannose-polysaccharides. At this point, therefore, it was felt that these data could be employed as a tool in the structural analysis of oligosaccharides, in which certain structural features are left in doubt by more orthodox methods. Two such studies will be described briefly.

The first involved a mannosyl-glycerol, obtained by alkaline hydrolysis from *M. lysodeikticus* (12, 13). This compound was found to inhibit the dextran-concanavalin A precipitation reaction to the same extent as methyl α -D-mannopyranoside (cf. the equal inhibition activities of 2-O- α -D-glucopyranosyl-D-glycerol and methyl α -D-glucopyranoside). The α -configuration was therefore assigned to the mannosyl-glycerol linkage. Subsequent investigations have supported this assignment (13).

Second, two tetrasaccharides, which on the basis of their mode of preparation from panose (14) could be assigned either structure H or L (Fig. 3), were tested as inhibitors in the dextran-concanavalin A system. One of the tetrasaccharides inhibited the reaction (1.6 μ moles for 50% inhibition; Fig. 2) close to the value found for maltose and was therefore tentatively assigned structure H, whereas the second tetrasaccharide was assigned structure L because it displayed an

inhibition (3 μ moles for 50% inhibition, Fig. 3) similar to the inhibition of trisaccharide J.

It is suggested that, with the examination of a greater number of model compounds, this method may find a general application in the structural analysis of saccharides containing D-glucose, D-mannose, 2-acetamido-2-deoxy-D-glucose, and certain of their derivatives.

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