

PROTEIN-CARBOHYDRATE INTERACTION.

PART XXII. A CHEMICALLY-SYNTHESIZED D-MANNAN AND THE INTERACTION OF SOME SYNTHETIC D-MANNANS WITH CONCAVALIN A

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ABSTRACT

1,6-Anhydro- β -D-mannopyranose was polymerized in the presence of chloroacetic acid. A high-molecular-weight fraction insoluble in 80% ethanol was isolated and studied. Periodate-oxidation studies showed the polymer to contain 42% of (1 \rightarrow 6)-like linkages, 36% of (1 \rightarrow 4)- or (1 \rightarrow 2)-like linkages, and 22% (1 \rightarrow 3)-like linkages. End-group analysis gave $\overline{d.p.} = 113$. The synthetic D-mannan reacted vigorously with concanavalin A, indicating that it was a highly branched polymer containing multiple α -D-mannopyranosyl residues at chain ends. A synthetic D-mannan obtained by the polymerization of D-mannose in the presence of phosphorous acid gave a much weaker reaction with concanavalin A, and two synthetic, linear polymers failed, as expected, to form a precipitate with this plant protein.

INTRODUCTION

The chemical synthesis of polysaccharides has stimulated a good deal of interest, especially during the past ten years¹. Recently, methods have been developed for the stereospecific polymerization of sugar derivatives to give polymers of defined linkage-type²⁻⁶.

In view of our interest in protein-carbohydrate interaction⁷⁻¹¹ we have prepared a synthetic D-mannan and have investigated its interaction with concanavalin A. This plant protein forms a precipitate with biopolymers containing terminal, non-reducing α -D-glucopyranosyl, α -D-mannopyranosyl, and β -D-fructopyranosyl residues. We have suggested that concanavalin A, although more limited in its specificity compared with certain anticarbohydrate antibodies, can be considered as belonging to this class of "protein reagents", all of which may be used for the detection and preliminary characterization of various structural features present in complex carbohydrates. (See Heidelberger¹² for a review.)

This report describes the acid-catalyzed, addition polymerization of 1,6-anhydro- β -D-mannopyranose and the isolation of a high-molecular-weight D-mannan

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for structural characterization. The interaction of concanavalin A with the described D-mannan, as well as with synthetic D-mannans prepared by several other procedures, was also studied.

MATERIALS AND METHODS

1,6-Anhydro- β -D-mannopyranose was prepared from the corresponding 2,3-isopropylidene acetal according to the procedure of Knauf *et al.*¹³; m.p. 209–210°, $[\alpha]_D^{20} - 127^\circ$ (*c* 1, water). Lit.¹³ m.p. 210–211°; $[\alpha]_D - 127^\circ$ (*c* 1.5, water).

Three samples of synthetic D-mannans were the gifts of Dr. Peter T. Mora¹⁴, Dr. Conrad Schuerch, and Professor P. S. O'Colla¹⁵. Chloroacetic acid was a product of Distillation Products Industries, Rochester, New York. Concanavalin A was prepared according to the method of Agrawal and Goldstein¹⁶. Glycerolde hydrogenase was purchased from Worthington Biochemical Corporation, Freehold, New Jersey.

EXPERIMENTAL

Polymerization of 1,6-anhydro- β -D-mannopyranose. — Vacuum-dried 1,6-anhydro- β -D-mannopyranose (4.0 g) and chloroacetic acid (95 mg) were finely powdered in a dry mortar. The resulting mixture was distributed equally in 8 test tubes and the tubes were immediately sealed. The tubes were heated in a glycerol bath (126–129°) for 5.3 h, to give a yellow glass that fractured upon cooling. The tubes were opened and water (5 ml) and solid sodium hydrogen carbonate (25 mg) were added to each tube. After 2 h with occasional stirring the yellow solutions from the 8 tubes were combined and dialyzed overnight against distilled water as follows: 1 liter of water (15 h, dialyzate was brownish yellow); 1 liter of water (8 h, dialyzate was light brownish-yellow); 1 liter of water (39 h, dialyzate was deep yellow); 1 liter of water (7 h, dialyzate was colorless). The 4 dialyzates were concentrated individually, the weights of the residue in each case being (1) 1.82 g, (2) 0.399 g, (3) 0.941 g, and (4) 0.068 g.

The indiffusible polymer solution from the dialysis sac was transferred to a beaker and the volume adjusted with water to 100 ml. Absolute ethanol (567 ml) was added dropwise with stirring, making the solution 85% with respect to ethanol. The resulting, turbid solution was centrifuged and the residue was washed successively, twice each with 85% ethanol, absolute ethanol, ether, and finally with petroleum ether (b.p. 30–60°) discarding the supernatant liquid each time. The residue was dried *in vacuo* to give a brown solid (polymer I, 1.219 g). Estimation of carbohydrate by the phenol-sulfuric acid procedure¹⁷ revealed polymer I to contain 23.5% of moisture. The net yield was 0.933 g (26%).

Polymer I (0.90 g) was dissolved in water (50 ml) and absolute ethanol (200 ml) was added dropwise with stirring, thus bringing the concentration of ethanol to 80%. The turbid solution was centrifuged and the residue (polymer II) washed successively with 80% ethanol, abs. ethanol, acetone, ether, and petroleum ether, and dried *in vacuo* over calcium chloride. Polymer II (0.726 mg) was obtained as a yellowish-brown powder (corrected for 12.9% water content by the phenol-sulfuric acid method¹⁷).

Paper-chromatographic analysis of polymers and dialyzates. — The four dialyzates from polymer I as well as polymers I and II were chromatographed [10:4:3 (v/v) ethyl acetate-pyridine-water] against D-mannose. Sugars were visualized using the silver nitrate-sodium hydroxide reagent¹⁸. Dialyzates I and II had components migrating with the mobility of mannose and higher saccharides, dialyzate III had only higher saccharides. Dialyzate IV, as well as polymers I and II, were immobile in this solvent system.

Periodate oxidation of polymer II. — To a solution of polymer II (132 mg) in cold water (10 ml) was added ice-cold 0.2M sodium periodate solution (25 ml) and the volume was adjusted to 50 ml. A control solution (without polymer) was prepared similarly. Both blank and reaction solutions were kept at 5° in the dark. Periodically, aliquots were removed and titrated for periodate consumption and formic acid liberation by using standard thiosulfate solution to titrate the iodine liberated upon addition of potassium iodide. On a dry-weight basis, the periodate consumption and formic acid liberation per D-mannosyl residue was 1.20 moles/mole and 0.42 moles/mole, respectively, after 69 h (constant).

Reaction of synthetic D-mannan with Fehling solution. — Addition of Fehling solution to an aqueous solution of the synthetic D-mannan (10 mg of polymer II in 1 ml of water) gave an immediate precipitate.

End-group analysis¹⁹ of polymer II. — To a solution of polymer II (26.7 mg, dry weight) in water (1 ml) was added sodium borohydride (30 mg) and the reaction was allowed to proceed for 49 h at 23°. The reaction mixture was adjusted to pH 5.5 (acetic acid), cooled to 5° and cold 1.0M sodium metaperiodate solution (2.5 ml) was added. The reaction volume was adjusted to 25 ml and the reaction flask was kept at 5° in the dark. A control solution containing all components except polymer II was prepared similarly. At various time intervals, aliquots (2 ml) were removed and analyzed for formaldehyde²⁰ giving the following results.

Time:	(h)	2	24	46	120	169	
HCHO:	(μ g)	6.25	37.5	63.1	78.1	78.1	(2.6 μ moles)

When polymer II (26.7 mg, 165 μ moles) was oxidized directly with sodium metaperiodate (no prior reduction with borohydride) and analyzed for liberation of formaldehyde as above, there was obtained 34.3 μ g (1.14 μ moles) of formaldehyde after 169 h (constant).

If it is assumed that the difference in the amount of formaldehyde produced upon periodate oxidation of borohydride-reduced polymer II and native polymer II represents the proportion of formaldehyde liberated from the reducing-end alditol residue, then the $\overline{\text{d.p.}}$ can be calculated from the formula¹⁹:

$$\overline{\text{d.p.}} = \frac{Y(30 \times n)}{162X},$$

in which Y = wt (g) of polymer; X = wt (g) of HCHO (mol wt 30); n = moles of

HCHO liberated from the reducing end-group ($n = 2$ for a C-3 or a C-4 linked end-group, $n = 1$ for a C-2, C-5 or C-6 linked end-group; 162 = wt (g) of monomeric residue.

Since the nature of the end-group linkage is unknown, it was further assumed that $n = 1$; and thus $\overline{d.p.} = 113$.

Studies on periodate-oxidized, reduced polymer II. — Polymer II (45 mg, dry weight) was oxidized with 0.1N sodium metaperiodate for 69 h at 5° as above. The reaction mixture was neutralized with barium carbonate, filtered and the filtrate and washings were concentrated. The residue was dissolved in water (5 ml) and the solution added to a solution of sodium borohydride (97 mg) in water (1 ml). After 4.5 h, an additional portion of sodium borohydride (100 mg) was added and the reaction mixture was kept for 20 h. The reaction mixture was neutralized (acetic acid), treated with Amberlite IR-120 (H⁺), and filtered. The filtrate was evaporated and anhydrous methanol was evaporated several times from the residue to remove methyl borate.

A solution of the residue in 1.25N sulfuric acid (3 ml) was heated for 3 h on a boiling water bath. The reaction mixture was cooled, treated with Amberlite IR-45 anion-exchange resin, and filtered into a 25-ml volumetric flask. The washings from the resin were added and the volume was adjusted to 25 ml. Paper-chromatographic analysis of the hydrolyzate [4:5:1 (v/v) butyl alcohol-ethanol-water] showed the presence of glycerol, erythritol, and mannose (alkaline silver nitrate spray-reagent¹⁸).

Mannose in the hydrolyzate was determined by quantitative paper chromatography (48 h in above solvent system) with the phenol-sulfuric acid method¹⁷. Found: 9.68 mg.

Erythritol was determined, after elution from the same chromatogram, by the method of Lambert and Neish²⁰. Found: 5.46 mg.

Glycerol was determined by an enzymic procedure with glycerol dehydrogenase²¹. Found: 10.5 mg.

Precipitation reaction between concanavalin A and the synthetic D-mannan. — A quantitative precipitation study was conducted between concanavalin A (36.9 μ g N)

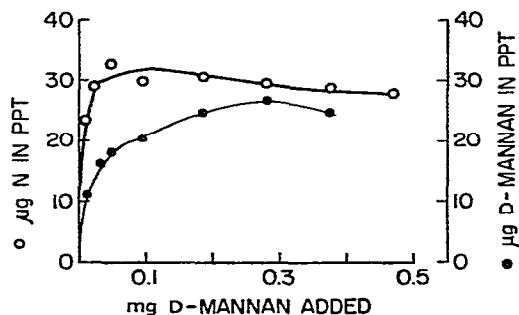


Fig. 1. Quantitative precipitation curve of synthetic D-mannan (polymer II) with concanavalin A. The total amount of synthetic D-mannan in the precipitate is also illustrated. Concanavalin A, 36.9 μ g N.

and increasing quantities of polymer II (0.01–0.5 mg), as described previously⁹. The carbohydrate content at each stage of the precipitation curve was also determined⁹ and the results are presented in Fig. 1.

By the procedure of So and Goldstein⁹, the solubility of the concanavalin A-polymer II D-mannan was determined to be 0.3 $\mu\text{g N/ml}$.

Interaction of the synthetic D-mannan prepared by Mora *et al.*¹⁴ with concanavalin A (48.3 $\mu\text{g N}$) was studied. The precipitation curve is presented in Fig. 2.

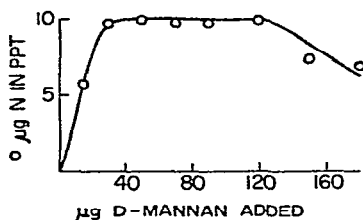


Fig. 2. Quantitative precipitation curve of synthetic D-mannan (Mora¹⁴) with concanavalin A (48.3 $\mu\text{g N}$).

The linear D-mannan from Dr. Schuerch was tested at levels of 40, 100, 150, 200, 300, and 500 μg against concanavalin A (40 $\mu\text{g N}$ and 120 $\mu\text{g N}$) and failed to form a precipitate. The synthetic D-mannan prepared by O'Colla and Lee¹⁵ also failed to react with concanavalin A (5, 15, 25, 50, 250, and 500 μg D-mannan against 40 μg and 120 μg of concanavalin A nitrogen, respectively).

DISCUSSION

Addition polymerization of 1,6-anhydro- β -D-mannopyranose in the presence of chloroacetic acid²² gave rise to a yellow glass from which a fraction insoluble in 80% ethanol was isolated in 20% yield and selected for study. The polymer was non-dialyzable, immobile on paper chromatograms, and precipitated readily upon addition of Fehling solution. It consumed 1.20 moles of periodate and liberated 0.42 molar proportions of formic acid per D-mannosyl residue. These results correspond to the presence of 42% (1 \rightarrow 6)-like linkages, 36% (1 \rightarrow 4)- or (1 \rightarrow 2)-like linkages, and 22% (1 \rightarrow 3)-like linkages. Borohydride reduction of the periodate-oxidized D-mannan followed by complete acid hydrolysis afforded glycerol (53 mole %), erythritol (21 mole %), and D-mannose (26 mole %).

Molecular-weight determination was conducted by an end-group procedure¹⁹ which involved determining the quantity of formaldehyde liberated upon periodate oxidation of the native and the borohydride-reduced polymer. This method gave $\bar{d.p.}$ 113, assuming that the reducing-end D-mannosyl residue liberated one mole of formaldehyde [i.e. $n = 1$ (ref. 19)].

In this connection it is noteworthy that the native polymer liberated 1 mole of formaldehyde for every 148 D-mannosyl residues, suggesting the presence of D-mannofuranosyl residues unsubstituted at C-5 and C-6. Such residues were reported

for a D-glucan synthesized by Mora *et al.*²³⁻²⁵ and analyzed by Dutton and Unrau²⁶. Dutton and Unrau have also demonstrated the presence of furanose residues in a synthetic D-mannan²⁷.

These results point to a complex, randomly-polymerized structure containing a wide variety of glycosidic linkages. The presence of a substantial proportion of periodate-resistant D-mannose residues indicates the existence of (1→3)- or multiply-linked [e.g. (1→2) and (1→4)] mannosyl residues. The high proportion of glycerol attests to the presence of a substantial number of (1→6)-linked residues and/or terminal D-mannopyranosyl residues.

Although periodate-oxidation studies give no information concerning the branching frequency of the polymer, it is probable that this synthetic D-mannan is a highly branched structure; in fact its interaction with concanavalin A is completely consistent with such a suggestion.

The interaction of concanavalin A with the synthetic D-mannan (polymer II) is presented in Fig. 1. The curve is quite typical of polysaccharides reactive with concanavalin A, rising sharply to a maximum (48 μ g of D-mannan added); at this point, 90% of the concanavalin A added was found in the precipitate. The solubility of the precipitate (0.3 μ g N) is characteristic of highly branched α -D-mannans¹¹. This interaction of polymer II with concanavalin A is strong evidence for the presence of many terminal, nonreducing α -D-mannopyranosyl residues.

On the other hand, the D-mannan obtained by polymerization of D-mannose in the presence of phosphorous acid¹⁴ gave a much weaker reaction with concanavalin A (Fig. 2) precipitating only 21% of the protein at the point of maximum precipitation. In this example there are apparently far fewer sites available for interaction with concanavalin A. Mora *et al.*¹⁴ commented that this polymer appears to be less branched than their D-glucan, and attribute this difference to the lower temperature and shorter heating cycle employed in its preparation.

The (1→6)- α -D-mannopyranan²⁸ ($[\alpha]_D +122^\circ$ in methyl sulfoxide, prepared by Schuerch by the same procedure^{3,4} he employed in the synthesis of an (1→6)- α -D-glucopyranan) gave, as expected, no reaction with concanavalin A; nor did the synthetic D-mannan prepared by O'Colla and Lee¹⁵ by the melt polymerization of 1,2,3,4-tetra-*O*-acetyl- β -D-mannopyranose in the presence of zinc chloride. (Chemical investigation showed this polymer to be mainly an α -D-(1→6)-linked polymer having a \bar{P}_n of 10.) These results confirm the linear nature of these latter two polymers.

Diffusion studies on agar gel with the synthetic D-mannan (polymer II) described in this investigation have already been presented⁸. The relatively broad precipitation band formed when concanavalin A and polymer II interacted indicate the synthetic D-mannan to be quite polydisperse. Furthermore, the closeness of the precipitation band to the well containing concanavalin A, relative to the position of a precipitation band (very sharp) between yeast D-mannan (from *Saccharomyces cerevisiae*) and concanavalin A, also indicates the synthetic polymer to be a mixture of low-molecular-weight species; end-group analysis as performed in this study supports this view.

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