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# Protein–Carbohydrate Interaction

## XVI. The Interaction of Concanavalin A with Dextrans from *L. mesenteroides* B-512-F, *L. mesenteroides* (Birmingham), *Streptococcus bovis*, and a Synthetic α-(1 → 6) -D-Glucan.[1, 4]

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The precipitation reaction between concanavalin A, the jack-bean hemagglutinin and several dextrans has been examined by means of the quantitative precipitin technique. A synthetic linear α-(1 → 6)-D-glucan did not form a precipitate with concanavalin A, demonstrating that only branched α-D-glucans precipitate with the protein.

The dextran from *Streptcoccus bovis* produced a typical precipitation curve with concanavalin A and gave the disaccharide nigerose when subjected to acetolysis. On the basis of these data it is suggested that this dextran, hitherto believed to be linear, is in fact a branched polymer.

The concanavalin A precipitation curves of the dextrans from *Leuconostoc mesenteroides* NRRL B-512-F and *Betacoccus arabinosaceous* (*L. mesenteroides*) Birmingham strain were compared with the *S. bovis* dextran and an attempt has been made to correlate these data with the molecular structures of these dextrans. The differences in the solubility of the dextran-concanavalin A precipitates and the relative ease of inhibition of these systems in the presence of hapten inhibitor are also discussed.

Previous studies in this series have been concerned with the isolation, purification (1, 2) and characterization (3, 4) of concanavalin A (the jack-bean hemagglutinin) and its interaction with simple (5–8) and complex carbohydrates (9–13). We have observed that concanavalin A exhibits the properties of a multivalent (recently shown in this laboratory to be bivalent (14)) antibody-like protein which interacts to form a precipitate with a specific group of ramified polysaccharides (5, 6, 9–13) which contain α-D-glucopyranosyl, α-D-mannopyranosyl or β-D-fructofuranosyl residues at their chain ends.

Quantitative hapten inhibition studies (5–7) with mono- and oligo-saccharides revealed the specificity of the protein-combining sites to be directed toward the C-3, C-4, and C-6 hydroxyl groups of the α-D-*gluco*- or *manno*-pyranose rings, with the latter being the preferred form. These studies also confirmed that it is primarily the terminal, non-reducing glycosyl residues of polysaccharide chain ends with which concanavalin A interacts, although it has been shown that in the case of the disaccharide sophorose (2-*O*-

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β-D-glucopyranosyl-D-glucose), it is the C-3, C-4, and C-6 hydroxyl groups of the *reducing* D-glucosido moiety with which the protein interacts (8). Our data also indicate that hydrogen bonds are importantly involved in stabilizing concanavalin A-carbohydrate complexes (6, 15).

Concanavalin A did not form a precipitate with several linear α-D-glucans (amylose, nigeran, pullulan and isolichenan (9)); however, all 23 dextrans which were tested for their capacity to interact with concanavalin A formed precipitates. These results prompted us to suggest that all of these dextrans were branched and, in fact, that it was possible that all naturally occurring dextrans were branched (9).

Presently we are examining the feasibility of using concanavalin A as a reagent for studying the fine structures of polysaccharides. This report is concerned with an interpretation of the relationship between polysaccharide structure and the results obtained from quantitative precipitation studies of concanavalin A with several dextrans: dextran *Leuconostoc mesenteroides* NRRL B-512-F (16) (the source of clinical dextran); dextran *Leuconostoc mesenteroides* (Birmingham)[5] (17); the dextran from *Streptococcus bovis* (18), reported to be a linear polymer; and a chemically synthesized α-(1 → 6)-D-glucopyranan (19) which has been shown by enzymic degradation (20) to contain a minimum of 98% α-(1 → 6)-D-glucosidic linkages.

## MATERIALS AND METHODS

Dextran NRRL B-512-F was a gift of Dr. Allene Jeanes, Northern Research and Development Service, U.S. Department of Agriculture; the dextran from *Leuconostoc mesenteroides* (Birmingham) was obtained through the courtesy of Dr. S. A. Barker, University of Birmingham, England; the dextran synthesized by *Streptococcus bovis* was a gift of Dr. H. Weigel, Royal Holloway College, England; and the synthetic α-(1 → 6)-D-glucopyranan was kindly provided by Dr. Conrad Schuerch, Syracuse University.

Methyl α-D-glucopyranoside and β-D-glucose penta-acetate were purchased from Pfanstiehl Laboratories, Inc., Waukegan, Illinois. Silica gel G was obtained from Brinkmann Industries, Westburg, New York. Authentic samples of β-nigerose octa-acetate and β-isomaltose octa-acetate were kindly provided by Dr. D. Horton, Ohio State University.

Concanavalin A was prepared by the Sephadex procedure of Agrawal and Goldstein (1, 2). Quantitative precipitation and solubility studies were conducted by the method described by So and Goldstein (12) and quantitative hapten inhibition analyses were performed as described previously (6).

Solvent systems employed in paper chromatography were: Solvent 1: butanol:ethanol:water (4:1:5, v/v, upper phase) (21); Solvent 2: ethyl acetate:pyridine:water (10:4:3, v/v) (22).

Acetolysis of the *S. bovis* dextran was conducted by the procedure of Suzuki and Hehre (23).

*Acetolysis of the dextran from* S. bovis. Dried dextran (387 mg) was suspended with stirring in cold acetic anhydride (4.0 ml). To this was added a mixture of cold acetic anhydride (2.4 ml) and concentrated sulfuric acid (0.8 ml). The reaction mixture was stirred in an iced water bath for 15 min, allowed to stand with occasional shaking in a water bath (43–54°) for 4 hr, and then at 35° for an additional 70 hr. The suspension (approximately 20% of the dextran remained insoluble) was poured into iced water (120 ml) and stirred for 15 min. Sodium bicarbonate was added slowly, with stirring, to pH 6. After extraction with chloroform (5 × 125 ml), the combined chloroform extracts were washed with 5% sodium bicarbonate solution (5 × 225 ml), water (3 × 150 ml), dried over anhydrous sodium sulfate and filtered. Thin-layer chromatography on Silica gel G with methanol-benzene (4:96, v/v) indicated a series of components with mobilities similar to standard β-D-glucose penta-acetate, β-nigerose octa-acetate and β-isomaltose octa-acetate, as well as components of lower mobility.

*Deacetylation of the acetolysis mixture.* The acetolysis mixture (in chloroform) was evaporated to a brown syrup, dried in a vacuum oven (40°) and weighed (725 mg). After dissolution in chloroform (6.0 ml), an aliquot (3.0 ml) was removed, mixed with additional chloroform (3 ml), methanol (2 ml), sodium methoxide solution (80 mg of sodium in 4 ml of methanol) and allowed to react under anhydrous conditions for 45 min. Evaporation to dryness followed by addition of water (15 ml) left a small

[5] This organism has been variously referred to as *Betacoccus arabinosaceous*, *Betacoccus arabinosaceous* (*Leuconostoc mesenteroides*), Birmingham strain and *Leuconostoc mesenteroides* NRRL B-1375 (Birmingham). In this paper, the dextran produced by this organism will be referred to as the "Birmingham dextran."

quantity of undissolved material. The solution was immediately neutralized (Amberlite IR-120-cation exchange resin, $H^+$ form) filtered and evaporated to a syrup which was dissolved in absolute ethanol. The evaporation was repeated and the syrup was dried (vacuum oven, 40°) and weighed (190 mg).

*Preparative paper chromatography of oligosaccharides.* An aqueous solution (1 ml) of the oligosaccharide-mixture was applied to Whatman 3 MM chromatography paper. The papers were irrigated (solvent 1) for 71 hours, and dried. Guide strips were cut, sprayed with alkaline silver nitrate reagent (24) and the areas corresponding to material of the same mobilities as nigerose (fraction A) and isomaltose (fraction B) were excised from the chromatogram. The saccharides were eluted with water and the solutions evaporated to clear syrups giving: fraction A (8.4 mg) $[\alpha]_D^{23}$ + 143° (c 0.84; $H_2O$); and fraction B, (12.6 mg).

Paper chromatographic examination of fractions A and B (in solvent systems 1 and 2) showed fraction B to have the same mobility as isomaltose and fraction A to migrate ($R_G$ 0.68, 0.71, respectively in the above solvent systems) similarly to nigerose ($R_G$ 0.69, 0.72) but differently from other α-glucobioses (kojibiose, $R_G$ 0.57, . . . . ; maltose, $R_G$ 0.68, 0.64; isomaltose, 0.46, 0.48).

*Qualitative paper electrophoresis.* Paper electrophoresis of fraction A (0.1 M sodium tetraborate, pH 10) for 7.5 hr at 100 V (23–32 mA) followed by drying of the electrophoretogram and development with *p*-anisidine spray reagent (25) revealed that it had a mobility identical with that of nigerose ($M_{maltose}$ 1.7) but considerably greater than that of maltose ($M_{maltose}$ 1.0).

*Acetylation of fraction A.* Fused sodium acetate (20 mg) and fraction A (6 mg) were dissolved in acetic anhydride (1.5 ml) with heating (85–125°) over a period of 0.5 hr. The solution was cooled, poured into iced water (10 ml) and stirred overnight at 5°. The aqueous solution was extracted with chloroform (3 × 15 ml) and the chloroform extract washed with water (3 × 25 ml) and evaporated to a straw-yellow syrup (8 mg). The syrup was dissolved in hot ethanol and upon prolonged cooling, produced a small quantity of crystalline material, m.p. 110–112° $[\alpha]_D^{23}$ + 77° (c 0.68; $CHCl_3$). Goldstein and Whelan (26) reported m.p. 152–153° and $[\alpha]_D^{23}$ + 83° (c 1.3; $H_2O$). Barker *et al.* (27) cited a melting point of 111–113° for β-nigerose octa-acetate.

*Periodate oxidation of dextran NRRL B-512-F and* S. bovis *dextran.* An aqueous solution of polysaccharide (100 mg/25 ml) was mixed with a solution of 0.35 N sodium metaperiodate (10.0 ml) and diluted with water to 50.0 ml. The reaction mixture was allowed to proceed in the dark, at 4° for 5 days. To determine periodate consumption, aliquots (1.5 ml) were withdrawn periodically, added to a solution containing 30% potassium iodide (2 ml), 2 N sulfuric acid (2 ml) and deionized water (10 ml). The browish-red solution was covered and allowed to stand in the dark for 5 min and titrated with standard sodium thiosulfate solution (0.109 N), utilizing starch as an indicator. The formic acid released during oxidation was determined periodically on aliquots (1.5 ml) removed from the reaction flask. These were added to a solution of ethylene glycol (0.5 ml) in deionized water (10 ml), stored in the dark for 25 min, and mixed with 30% potassium iodide (2 ml). The solutions were covered, allowed to stand in the dark for 5 min and titrated with standard sodium thiosulfate (0.011 N) to the first starch end-point.

## RESULTS

Quantitative precipitation curves for the interaction of concanavalin A with the three microbial dextrans examined in this study are shown in Figs. 1, 2, and 3. The *S. bovis* and B-512-F dextrans were far less reactive than dextran NRRL B-1355-S, the dextran employed in our assay system (12). Thus, whereas dextran B-1355-S precipitated at the point of maximum precipitation, 41 (98%) of the 42 μg of concanavalin A nitrogen in the incubation mixture, it was necessary to add substantially more concanavalin A (approximately 139 μg N) to obtain a reliable precipitation curve for the *S. bovis*

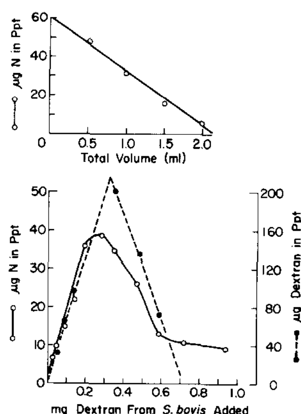


FIG. 1. Quantitative precipitation curve of the *S. bovis* dextran with concanavalin A. The total amount of dextran in the precipitates is also illustrated. Concanavalin A, 139 μg of nitrogen. The solubility of the concanavalin A-*S. bovis* dextran is also demonstrated.

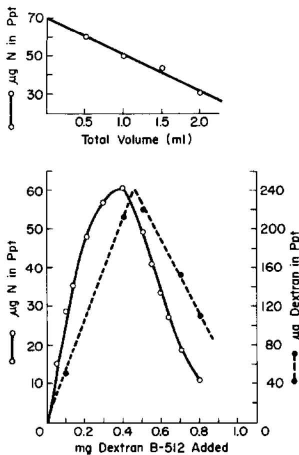


FIG. 2. Quantitative precipitation curve of dextran B-512-F with concanavalin A. The total amount of the dextran in the precipitates is also illustrated. Concanavalin A, 155 μg of nitrogen. The solubility of the concanavalin A-B-512-F dextran is also demonstrated.

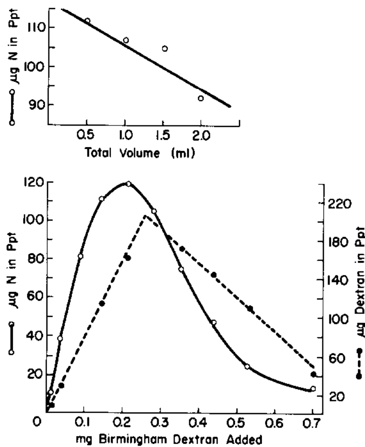


FIG. 3. Quantitative precipitation curve of the Birmingham dextran with concanavalin A. The quantity of the dextran in the precipitates is also illustrated. Concanavalin A, 144 μg of nitrogen. The solubility of the concanavalin A-Birmingham dextran is also demonstrated.

dextran, which precipitated only 39 μg (28%) of the nitrogen added. Similarly, dextran B-512-F maximally precipitated only 60 μg (39%) of the 155 μg N added. In contrast, the dextran elaborated by *L. mesenteroides* (Birmingham) was considerably more reactive, precipitating 120 μg (83%) of the 144 μg concanavalin A nitrogen added to the reaction mixture.

Table I demonstrates the unreactivity of the synthetic α-(1 → 6)-D-glucan. Even at a level of 141 μg N (3.5 times that at which dextran B-1355-S precipitates 98% of the added concanavalin A) (12) the synthetic polyglucose did not precipitate concanavalin A. This is of course not surprising inasmuch as this polysaccharide is believed to be a linear polymer (19,20) and hence incapable of forming a three-dimensional lattice with concanavalin A.

Only about 50% of the *S. bovis* and B-512-F dextrans added were found in the precipitates at the point of maximum nitrogen precipitation. In the case of the Birmingham dextran this figure is considerably higher, being approximately 79%. It will be recalled (12) that concanavalin A precipitated virtually all of the dextran B-1355-S added, up to and throughout the "equivalence zone."

The solubility of the dextran-concanavalin A precipitates (at the point of maximum nitrogen precipitation) is a very revealing parameter. Compared to the dextran B-1355-S-concanavalin A precipitate which had a solubility of 1.5 μg N per ml (12), the solubility of the *S. bovis* dextran-concanavalin A precipitate was 28 μg N per ml (Fig. 1), that of dextran B-512-F-concanavalin A precipitate, 19 μg N per ml (Fig. 2) and that of the Birmingham dextran-concanavalin A precipitate, 11 μg N per ml (Fig. 3). The significance of these solubility differences in terms of molecular structure will be discussed below.

Quantitative hapten inhibition studies employing the three dextrans as the precipitating polysaccharide are shown in Fig. 4. The extent (percentage inhibi-

TABLE I

INTERACTION OF CONCANAVALIN A WITH A SYNTHETIC, LINEAR α-(1 → 6)-D-GLUCAN (19)

| Concanavalin A (μg nitrogen) | Dextran (mg) | Nitrogen in precipitate (μg) |
|---|---|---|
| 141 | 0.45 | 0.1 |
| 141 | 0.91 | 0 |
| 141 | 1.36 | 0 |
| 141 | 1.81 | 0 |

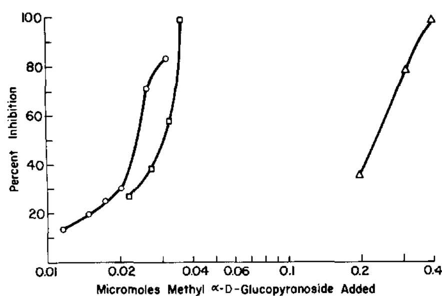


FIG. 4. Inhibition of precipitation of concanavalin A-dextran interaction by methyl α-D-glucopyranoside. (○), *S. bovis* dextran; (□), dextran B-512-F; (△), Birmingham dextran.

tion) to which methyl α-D-glucopyranoside inhibited precipitation of the respective systems is plotted against the concentration of this glycoside in micromoles on a logarithmic scale. It is apparent that the *S. bovis* and B-512 dextran systems are much more readily inhibited than the concanavalin A-Birmingham dextran system, the quantities of methyl α-D-glucopyranoside required to produce 50% inhibition being 0.024, 0.03, and 0.22 μmoles, respectively.

The similarity in the structures of the dextrans from *S. bovis* and *L. mesenteroides* NRRL B-512-F is shown by the similar behavior of both polysaccharides during controlled periodate oxidation. Thus, both glucans released 0.95 moles of formic acid/mole glucosyl residue and consumed 2.01 (*S. bovis*) and 1.90 (B-512-F) moles of periodate/mole glucosyl residue after 8.6 and 8.3 hr of reaction time, respectively. These results may be compared to those of Rankin and Jeanes (28) who reported that dextran B-512-F, during oxidation, released 0.95 moles of formic acid and consumed 1.94 moles of periodate/mole glucosyl unit. Similarly, Bailey (18) reported a value of 2.00 moles of periodate consumed/mole of glucosyl residue during the oxidation of the dextran from *S. bovis*.

A comparison of the properties of authentic nigerose with the nigerose isolated by acetolysis from the *S. bovis* dextran established the identity of the isolated material.

## DISCUSSION

The present study confirms our previous investigations by: (1) demonstrating that a synthetic linear α-(1 → 6)-D-glucan (17) containing a minimum of 98% α-(1 → 6)-D-glucosidic linkages as deduced by enzymatic degradation (20) did not form a precipitate with concanavalin A, even at protein levels which brought about the precipitation of all the naturally occurring dextrans which we have examined; and (2) showing that the dextran from *S. bovis*, reported by several criteria to be linear (18) is almost certainly branched.

From an examination of the polyglucoses produced by several strains of rumen *S. bovis* grown on sucrose and $HCO_3^-$ ion, Bailey (18) stated these polysaccharides are unbranched α-D-glucans possessing solely α-(1 → 6)-D-glucosidic linkages. These conclusions were based primarily on: (1) partial acid hydrolysis studies in which isomaltose was the only disaccharide identified; (2) controlled oxidation with sodium periodate, which produced results that were very close to those expected for a linear (1 → 6)-glucan and (3) the absence of the infrared absorption band (12.6 μ) believed to be characteristic of α-(1 → 3)-linkages (16,29).

Inasmuch as concanavalin A formed a precipitate with the *S. bovis* dextran, an indication of branching in the polymer, we undertook an examination of the structure of this polysaccharide, by techniques other than those used by Bailey (18). It is well documented that acid hydrolysis preferentially destroys α-(1 → 2)-, α-(1 → 3)-, and α-(1 → 4)-D-glucosidic bonds as compared to α-(1 → 6)-linkages (30). Therefore, a glucan containing only small proportions of non-α-(1 → 6)-D-glucosidic units would not be expected to yield, upon partial acid hydrolysis, disaccharides characteristic of these structural features, i.e., kojibiose, nigerose, and maltose. However, acetolysis has been shown to cleave α-(1 → 6)-linkages considerably more readily than linkages in-

volving secondary hydroxyl groups of glycosyl residues (23, 26, 31–35).

Indeed, nigerose, in a minimum yield of 4%, was isolated from a deacetylated acetolysis mixture of the *S. bovis* dextran. The properties of this disaccharide compared very favorably with an authentic sample of nigerose and this disaccharide undoubtedly represents a structural feature of the polysaccharide. Although it is probable that a portion of the nigerose isolated represents branch-point linkages, it is known that linkages other than those involving α-(1 → 6)-D-glucopyranosidic bonds occur in linear portions of dextran molecules; e.g., we have evidence that dextran B-1355-S contains α-(1 → 3)-glucosidic linkages in the linear portion of the dextran molecule (cf. Ref. 36).

In a comparison of the periodate oxidation of the dextrans from *S. bovis* and *L. mesenteroides* NRRL B-512-F (which has been shown by methylation studies to possess 96% α-(1 → 6)- and 4% α-(1 → 3)-D-glucosidic bonds) (37), it is interesting to note that similar if not identical results were obtained with regard to both periodate consumption and release of formic acid. It is apparent, therefore, that acetolysis is a considerably more productive technique for obtaining evidence for the existence of a small proportion of α-(1 → 3)-D-glucosidic linkages in polysaccharides. Jeanes *et al.* (16) regarded 2% as the limit of detection of such units by measurement of periodate consumption and formic acid release. Suzuki and Hehre (23) in an examination of the acetolysis of a variety of dextrans noted that ten preparations, which yielded from 0.4–2.9% nigerose, showed no (1 → 3)-like residues by periodate oxidation. These workers also reported that acetolysis of dextran NRRL-B-742-L yielded 1.1% nigerose. This is especially interesting inasmuch as this polysaccharide has been regarded as essentially free of (1 → 3)-linkages and therefore was employed as the zero standard in infrared spectrophotometric analysis for (1 → 3)-linkages in polysaccharides (absorption band at 12.6 μ) (16, 29).

The precipitation curves (Figs. 1, 2, and 3) for the three microbial dextrans examined are typically bell-shaped. The Birmingham dextran was the most reactive of the three in terms of maximum concanavalin A nitrogen precipitated (83%) and carbohydrate brought down in the precipitate (79%). If one corrects for the solubility (11 μg N/ml) of the precipitate (neglecting the two washings of the precipitate), it is possible to account for 92% of the concanavalin A in the incubation mixture.

On the other hand, the *S. bovis* and B-512-F dextrans precipitated, at the point of maximum precipitation, only 28 and 39%, respectively, of the concanavalin A nitrogen and less than 50% of the dextran present in the initial reaction mixtures.

Although we have accumulated considerable information on the factors which influence polysaccharide-concanavalin A interaction, i.e. molecular weight of the polysaccharide, frequency and nature of the branching, character of the linkage joining the terminal, nonreducing glycosyl unit to the succeeding (subterminal) unit, i.e. (1 → 6), (1 → 4), etc. and number of units in the exterior chains, there is still much to learn.

Nevertheless it is possible to make some interpretations of these data in terms of the molecular structure of the dextrans. The dextran elaborated by *L. mesenteroides* (Birmingham) was shown by methylation studies (37) to have a branch point for every 6 to 7 D-glucosyl residues; furthermore, the branch points were shown to arise from (1 → 3)-glucosidic linkages and it was calculated that more than 18% of all branches consist of a single glucosyl unit (38–40). This makes it approximately three times more ramified than B-512-F dextran, which contains one branch for every 23 units (37). Nothing is known concerning the extent of branching of the *S. bovis* dextran. It is to be noted that the more highly branched Birmingham dextran is the most reactive of the three dextrans with regard to its capacity to precipitate concanavalin A. The solubility of the concanavalin A-

dextran precipitates also is lowest in the case of the Birmingham dextran (Fig. 3). Furthermore, the concanavalin A-Birmingham dextran system is the most difficult to inhibit (Fig. 4) in the presence of methyl α-D-glucopyranoside, 0.22 μmoles being required for 50% inhibition of precipitation compared to 0.025 and 0.030 μmoles of this glycoside to inhibit the B-512-F dextran- and the *S. bovis* dextran-concanavalin A systems, respectively.

It is believed that at least 70% of the branches in dextran B-512-F are only one unit long (36). It might be expected that the concanavalin A combining sites (which can accommodate one α-D-glucosyl residue) would experience considerable hindrance in approaching a single glucosyl stub extending from a polysaccharide chain. One piece of data which supports this view is the inhibition data on 4,6-di-(α-D-glucopyranosyl)-D-glucose (7) (the branch-point trisaccharide of glycogen and amylopectin). It was considered that this trisaccharide by virtue of its two non-reducing α-glycosyl units would act as a "double-headed" inhibitor. In fact, it was shown to be somewhat less active than maltose, this being attributed to a steric effect; i.e., one can regard this substance 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 moiety. In either case the large α-glucosyl substituent would be expected to offer hindrance to binding by the protein.

On the basis of the reactivity of the *S. bovis* dextran with concanavalin A, and the presence of some α-(1 → 3)-linkages as deduced from the isolation of nigerose, we conclude that this dextran is in many respects similar to dextran B-512-F and probably is branched to a limited extent. The high solubility of the concanavalin A-*S. bovis* dextran precipitates and the great ease with which this system is inhibited support this conclusion.

We are presently investigating the interaction of concanavalin A with a large number of dextrans in the hope of defining further the structural parameters involved in polysaccharide-concanavalin A interaction.

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