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Specific Binding of Concanavalin A to Cross-Linked Dextran Gels

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Concanavalin A, a globulin found in the jack bean, agglutinates certain animal erythrocytes (Sumner & Howell, 1936). This protein also forms a precipitate with glycogen, amylopectin, dextrans and yeast mannan (Sumner & Howell, 1936; Cifonelli, Montgomery & Smith, 1956; Goldstein, Hollerman & Merrick, 1963, 1965a). The interaction of concanavalin A with carbohydrates is very specific, formation of a visible precipitate occurring only with branched polysaccharides that contain α -D-glucopyranosyl or α -D-mannopyranosyl residues at non-reducing chain ends (Goldstein *et al.* 1963, 1965a). We are studying this protein-carbohydrate interaction both as a tool for investigating the fine structure of polysaccharides and as a possible model for the antibody-antigen system.

Sumner & Howell's (1936) method of isolating concanavalin A from jack-bean meal involves the use of high concentrations of organic solvents (acetone and ethanol) and precipitation of the protein by dialysis of an NaCl extract of the meal in the presence of toluene. If toluene is omitted, concanavalin A is not readily precipitated even on prolonged dialysis. We have observed that the protein also aggregates in the presence of toluene; such aggregation may cause possible modification in the conformation and biological properties of concanavalin A. We have also confirmed Sumner's (1919) observation that $(\text{NH}_4)_2\text{SO}_4$ fractionation is unsatisfactory for the preparation of concanavalin A.

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Since the Sephadexes are a family of cross-linked dextrans, and various dextrans have been shown to be good precipitants for concanavalin A, we investigated the possibility of employing these substances as a means for purification of the protein. If concanavalin A should bind specifically to Sephadex, we would expect that unreactive extraneous proteins and other high-molecular-weight impurities could be removed by taking advantage of the molecular-sieve effects of these dextran gels. Results of the experiments described below fully demonstrate the feasibility of such an approach.

Methods. A 100g. portion of jack-bean meal (General Biochemicals Inc., Chagrin Falls, Ohio, U.S.A.) was suspended in 400ml. of aq. 0.15M-NaCl and stirred overnight in the cold (approx. 6°). The cloudy suspension was partially clarified by centrifugation at 18800g (12500rev./min. in the Sorvall RC-2 refrigerated centrifuge, SS-34 rotor) and the supernatant solution was saved. Extraction of the residue was repeated with 200ml. of 0.15M-NaCl, and after centrifugation under the same conditions the combined supernatant solutions were dialysed against running tap water.

The extract was dialysed successively against $(\text{NH}_4)_2\text{SO}_4$ solutions to give 0.20, 0.40, 0.60, 0.80 and 0.90 saturation. The residue and supernatant solutions obtained at each stage were examined for concanavalin A activity on the basis of a turbidimetric assay with dextran NRRL B-1355-S as the precipitant (Goldstein, Hollerman & Smith, 1965b).

Alternatively, the protein fractions were tested for activity against glycogen or other reactive polysaccharides by using the agar-gel diffusion technique (So & Goldstein, 1965). This assay was employed for protein fractionated on Sephadex G-200.

Sephadex columns were prepared in 1.0M-NaCl. A filter-paper disk was placed on the top of the gel bed. The protein solution in 1.0M-NaCl was applied to the column in the usual fashion. A reservoir containing 1.0M-NaCl was connected to the column and 8ml. fractions were collected. The fractions were monitored for protein by determining the extinction at 280m μ . When protein was no longer eluted with NaCl, a solution of 1.0M-NaCl containing glucose (0.1M) was added. The effluent was tested for glucose by the phenol-H₂SO₄ method (Dubois, Gilles, Hamilton, Rebers & Smith, 1956) and for protein as above. The protein so eluted was freed of glucose by dialysis against 1.0M-NaCl. The activity of the protein was assayed as above.

Results. Maximum concanavalin A activity was found to reside in the fraction that was precipitated with (NH₄)₂SO₄ between 0.4 and 0.6 saturation; this fraction was employed in all subsequent experiments.

With Sephadex G-25, all the protein was recovered in one fraction, indicating the lack of affinity of concanavalin A for dextran gels of such extensive cross-linking as Sephadex G-25.

With Sephadex G-200, the results indicate that concanavalin A is retained in the Sephadex G-200 bed. A fraction (approx. 50%) of the total protein was eluted with 1.0M-NaCl (Fig. 1); when tested it did not develop turbidity with dextran nor did it form a precipitation band with glycogen in gel-diffusion plates. The remainder of the protein was not eluted with 1.0M-NaCl, indicating that Sephadex G-200 provides sufficient sites for interaction with concanavalin A. That it is a specific interaction of concanavalin A with the appropriate sites on the Sephadex matrix is indicated by the ease of its elution with 0.1M-glucose, a competitive inhibitor of concanavalin A-polysaccharide interaction (Goldstein *et al.* 1965b). This protein fraction, after dialysis to remove glucose, was active in developing turbidity with dextran solutions and also formed a sharp precipitation band against rabbit-liver glycogen in agar diffusion plates.

With Sephadex G-100, G-75 and G-50, the elution patterns obtained were similar to that of Sephadex G-200. After non-reactive proteins had been removed the bound protein was displaced by glucose. The strong displacement by glucose is reflected in the sharpness of the protein peak.

Discussion. The Sephadexes are a family of dextran gels obtained by controlled cross-linking in the presence of epichlorohydrin (Flodin, 1962). The degree of cross-linking decreases in the order

Fig. 1. Chromatography of concanavalin A on a Sephadex G-200 column (3cm. × 50cm.). Fractions of volume 8ml. were collected. The arrow indicates the addition of glucose (0.1M). Fraction I was inert towards dextran and glycogen. Fraction II contained all the concanavalin A activity.

Sephadex G-25, G-50, G-75, G-100, G-200, and governs the extent to which the various Sephadexes retard solutes. The exclusion limit decreases with increase in cross-linking (Flodin, 1962; Pharmacia, 1963).

These experiments demonstrate that concanavalin A binds to Sephadex G-200, G-100, G-75 and G-50. Failure of the protein to bind to Sephadex G-25 suggests that the high degree of cross-linking in this gel eliminates the sites necessary for interaction.

Since the exclusion limit increases with increasing G-number of Sephadex, use of the most highly cross-linked dextran gel that still possesses the capacity to bind concanavalin A (Sephadex G-50) results in the most efficient removal of inert protein besides possessing the further advantage of faster flow rates, thereby shortening the time required for purification of the protein. After elution of concanavalin A with glucose and removal of the monosaccharide by dialysis, the cycle may be repeated to ensure complete removal of proteins other than concanavalin A. Sephadex G-200, the least cross-linked of the dextran gels, possesses more sites for interaction with concanavalin A than any other in the series and provides an ideal medium for concentrating solutions of concanavalin A.

The method described combines the molecular-sieve effect of Sephadex and the biological property of the protein. To our knowledge the only other instance in which Sephadex has been so used is in

the isolation and fractionation of human anti-dextran antibodies (Schlossman & Kabat, 1962). The affinity of the desired protein towards the dextran gel is exploited in both cases. Subsequent displacement by low-molecular-weight competitive inhibitors (haptens) yields a purified protein.

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Octadecenoic Acids in Sheep Rumen

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It is now well established that the rumen microflora is capable of efficient hydrogenation of unsaturated fatty acids (Reiser & Reddy, 1956; Shorland, Weenink, Johns & McDonald, 1957; Garton, Lough & Vioque, 1961). Though the rumen is the principal site of hydrogenation, there is evidence that hydrogenation takes place to a limited extent in other parts of the ovine digestive tract (Ward, Scott & Dawson, 1964). The dietary C_{18} polyenoic acids occur almost exclusively in the *cis* form, and although they are converted efficiently into stearic acid, the presence and possible accumulation of the *trans*-acids implies isomerization during hydrogenation (Garton, 1959). Kepler, Hirons, McNeill & Tove (1965) have reported that the rumen micro-organism *Butyrivibrio fibrisolvens* converts linoleic acid into a conjugated *trans* intermediate and then to elaidic acid *in vitro*. Our findings, made incidental to studies of the effects of fatty acids on the energy metabolism of the sheep and on the fermentation in their rumens, support these observations (Czerkawski, Blaxter & Wainman, 1965).

After control periods lasting for at least 3 weeks in which no infusions were made, emulsions of fatty acids were infused continuously into the rumens of sheep for similar periods. Samples of rumen fluid were withdrawn at the end of the control and infusion periods. Total lipids were extracted

with chloroform-methanol according to the method of Folch, Lees & Sloane-Stanley (1957); they were saponified, and the isolated fatty acids esterified with dry methanol in the presence of HCl (Kates, 1964). The methyl esters were separated on the Pye Argon Chromatograph with Apiezon L (Edwards High Vacuum Ltd., Crawley, Sussex) or polyethylene glycol adipate as the stationary phase (Farquhar, Insull, Rosen, Stoffel & Ahrens, 1959). The amounts of various components were estimated from the areas under the peaks.

The *cis*- C_{18} monoethenoic acid found in the rumen was identified as elaidic acid in the following way. It had exactly the same retention volume as oleic acid on the polyethylene glycol adipate column but emerged between oleic acid and stearic acid on the Apiezon column (retention volume 0.890 compared with 0.855 for oleic acid, assuming that stearic acid has a retention volume of 1.0). Its peak was eliminated by bromination; hydrogenation also led to its disappearance with a concomitant stoichiometric increase in the amount of stearic acid. The compound migrated between stearic acid and oleic acid on thin-layer plates of silicic acid impregnated with $AgNO_3$, and had the same R_F value as the reference sample of elaidic acid (Morris, 1964). Consequently in many instances, particularly when the proportions of oleic acid and elaidic acid were such as to give poor