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The Interaction between Polysaccharides and Other Macromolecules

VI. Further Studies on the Solubility of Proteins in Dextran Solutions

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The solubilities of cyanmethemoglobin and β-lactoglobulin in the presence of dextran were studied at high and low ionic strengths. The decreases in protein solubility in dextran solutions were very similar in both environments. The solubility of zein in 5 m urea decreased in the presence of the polysaccharide in a similar fashion. The results are in accord with the hypothesis that dextran sterically excludes a large volume of solvent from the proteins.

It was recently shown that the extent to which the presence of dextran decreased the solubility of various proteins increased with the size of the protein.[1] The results observed suggested the hypothesis that proteins were sterically excluded from a certain element of volume that could be considered the domain of the polysaccharide.

The initial investigation was confined to media containing high concentrations of ammonium sulphate so as to limit protein solubility to practical levels. The current study will show that the influence of dextran on the solubility of proteins in medium of low ionic strength is similar to that in the salting-out range. Zein, a protein which is insoluble in water but which dissolves in the presence of high concentrations of urea, is also demonstrated to be similarly affected by polysaccharides.

EXPERIMENTAL

Material

Dextran-500. This was the same as that used in the previous investigation.[1] Its weight-average molecular weight was 4.5×10^5.

Cyanmethemoglobin. This was prepared from 4000 ml of horse blood. The blood cells were spun down in a refrigerated centrifuge and then washed three times with 6000 ml

portions of 0.9 % sodium chloride. On completion of the washing, the cells were lysed by adding 1500 ml toluene [2] and stirring overnight in the cold. The suspension was then centrifuged at 23 000 × *g* in a Spinco preparative ultracentrifuge for 60 min. The water phase was treated with 300 ml of 9 % potassium ferricyanide and 2.3 % sodium cyanide [3] and left in the cold for 48 h. The reaction mixture was first dialysed against distilled water and subsequently against a phosphate buffer, pH 6.6, ionic strength 0.1. Cyanmethemoglobin was crystallized from this solution by slow addition of ethanol to a value of 8 % at 4°C. After recrystallization under the same conditions, the product was dissolved and dialysed against distilled water prior to experimental use. The logarithmic relationship between the solubility of this preparation and ammonium sulphate concentration was determined in the range from 2.7 m to 3.3 m and found linear. These measurements were made in the presence of 0.05 m phosphate buffer pH 6.6.

β-Lactoglobulin. The protein was prepared from pooled bovine skimmed milk according to the method of Palmer.[4] It was twice recrystallized by dialysis against distilled water of a solution with an original pH of 5.2. The crystals obtained in this manner were dissolved in 0.4 M sodium chloride. A high-molecular weight impurity in this stage of the preparation was removed by gelfiltration on a Sephadex G-200 column. After this treatment, the protein exhibited a single sedimentation boundary in the ultracentrifuge and had a molecular weight of 37 500 as determined by the approach to equilibrium method. The logarithm of its solubility was a linear function of the ammonium sulphate concentration in the range tested, *i.e.* 2.7 m to 3.15 m, in the presence of 0.14 m acetate buffer pH 5.2.

Zein (C grade) was obtained from California Corporation for Biochemical Research, Los Angeles 63, Calif. U.S.A. It sedimented in the ultracentrifuge as a single compound in a solution containing 80 % (v/v) of ethanol and 0.1 M sodium chloride.

Methods

Solubility of cyanmethemoglobin. Mixtures containing 1.75 g/100 ml of cyanmethemoglobin and zero to 15 g/100 ml of dextran were dialysed at 4°C for 24 h against large volumes of 2.6 m ammonium sulphate containing 0.05 m phosphate buffer pH 6.65. Volume changes occurring during dialysis were recorded to correct for changes in dextran concentrations. The precipitates that formed were spun down in a Spinco preparative ultracentrifuge at 25 000 × *g* for 30 min in a rotor precooled to 4°C. The concentration of protein remaining in the supernatant was estimated from the optical density at 540 mμ. The optical density of the protein solution was approximately 1.0 in the absence of dextran.

In another series of measurements, mixtures containing 17.5 g/100 ml of cyanmethemoglobin and varying concentrations of dextran were prepared in 0.2 m phosphate buffer pH 8.4. These were then dialysed for 72 h at 4°C against a large volume of 0.02 m phosphate buffer pH 6.65 containing 5 % (v/v) ethanol. The remainder of the experiment was performed according to the procedure described above. The optical density of the saturated protein solution was approximately 22 at 540 mμ in the absence of dextran.

Solubility of β-lactoglobulin. Solutions of protein were added to solutions containing varying concentrations of dextran in 0.4 m sodium chloride. The optical density of the mixtures was approximately 4 at 280 mμ. The mixtures were dialysed against 2.85 m ammonium sulphate and 0.14 m acetate buffer pH 5.2 for 48 h. The protein solubility was determined as above except for the fact that protein concentrations were estimated from the optical density at 280 mμ with appropriate dextran solutions used as blanks. The optical density of a saturated protein solution in the absence of dextran was approximately 2.

A corresponding series of experiments was performed on identical β-lactoglobulin-dextran mixtures dialysed for 100 h against an 0.001 m acetate buffer pH 5.2 before determining the solubility. In this instance the optical density of a saturated solution without dextran was approximately 1.

Solubility of zein. Stock solutions containing 8 % zein in 8.2 m urea and 15 % dextran in 6.45 m urea were prepared. Mixtures containing 2 % protein and varying concentrations of dextran were made up by adding appropriate quantities of stock solutions to a pure 6.45 m urea solution. After dialysis for 100 h in 5 m urea, the solubility of zein was

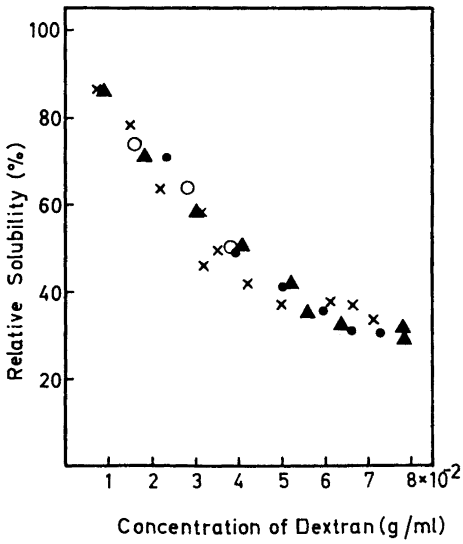

Fig. 1. The relative solubility of cyanmethemoglobin as a function of the dextran concentration. Three series of experiments were made in a medium of 2.6 m ammonium sulphate and 0.05 m phosphate buffer pH 6.65 (O, ▲, ●) and one in a medium of 0.02 m phosphate buffer pH 6.65 containing 5 % (v/v) of ethanol (×).

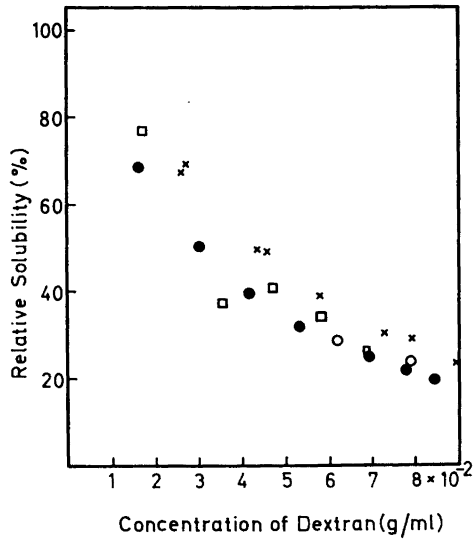

Fig. 2. The relative solubility of β-lactoglobulin as a function of the dextran concentration. Two series of experiments were made in a medium of 2.85 m ammonium sulphate and 0.14 m acetate buffer pH 5.2 (●, O) and two series in a medium of 0.001 m acetate buffer pH 5.2 (□, ×).

determined as in the previous experiments by measuring optical density at 280 mμ. Necessary dilutions were made with 6.45 m urea. Under these conditions a pure saturated zein solution was found to have an optical density of approximately 10.

RESULTS

The results are graphically summarized in Figs. 1, 2, and 3, where the relative decrease in solubility of a protein in the presence of dextran has been plotted as a function of dextran concentration. The solubility in the absence of the polysaccharide has been taken as 100 %.

The polysaccharide brought about a definite reduction in protein solubility in all the experiments performed. The ionic strengths of the media employed appeared to have little apparent influence on the effect of dextran on the solubility of either cyanmethemoglobin or β-lactoglobulin.

DISCUSSION

The present investigation is part of a general program of studies of the steric interactions between polysaccharides and other substances. The presence of polysaccharides or other polymers in a solution will lead to both a

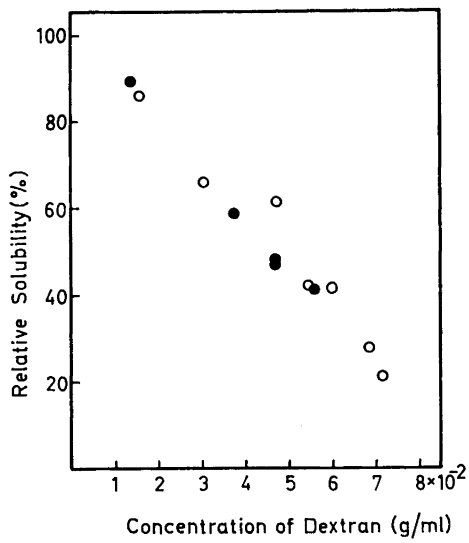

Fig. 3. The relative solubility of zein in the presence of dextran; two different experimental series in 5 m urea (●, ○).

restriction of the movement of other solutes [5] and to a restriction of available space for them.[6] Both phenomena can be regarded as manifestations of the spatial requirements of long chain polymers. The factors governing the spatial requirements of a polymer are many and include, *e.g.*, chemical structure, chainlength, branching, charge and polymer-solvent interaction.

Previous reports have discussed some of the consequences that result when a part of the volume of a system, normally available to a solute, is restricted. Decreasing the space for a solute increases its activity and leads to an increase in osmotic pressure.[7] This activity increase may be large enough to exceed the solubility limits of a solute and precipitation occurs.[1] In the latter of these investigations, it was shown that the presence of dextran decreased the solubility of proteins in accord with a hypothesis of steric exclusion. The current study was initiated to verify the hypothesis on a broader experimental basis. If the proposed mechanism is correct, then the relative decrease in solubility of proteins in the presence of polysaccharides should be independent of the solvent in which the solubility is measured, provided that the solvent does not change the size or shape of either the protein or polysaccharide. To test this, the solubility of two proteins have been compared in dextran solutions at both high and at low salt concentrations.

The proteins selected were cyanmethemoglobin and β-lactoglobulin. They are both easy to prepare in the relatively large scale required and reasonably soluble at low ionic strengths. In the case of cyanmethemoglobin it was found desirable to include a low concentration of ethanol to be able to work in a more practical solubility range. Lactoglobulin is known to be a heterogeneous protein [8,9] whose constituents vary somewhat with regard to their solubilities. This limits its usefullness. However, there are reasons to believe that this will not invalidate the conclusions made here. When the logarithm of the solu-

bility of the protein preparation was plotted *versus* ammonium sulphate concentration a linear plot was obtained within the concentration range in which the experiments were performed, indicating a solubility behaviour equivalent to that of a one-component system. The relative solubility curves depicted in Fig. 2 do not show any obvious break that would indicate more than one component. Furthermore, the solubility of the protein in the presence of dextran has been expressed relative to the solubility in the absence of polysaccharide, and it is expected that dextran should have the same relative effect on both β_1- and β_2-lactoglobulin for they are believed to have the same molecular weights.[9]

The results of the studies on cyanmethemoglobulin and lactoglobulin show that the effects of dextran on protein solubility are very similar at both high and low ionic strengths in verification of the hypothesis that the phenomenon involves a steric exclusion process. Zein, which is insoluble in water, behaves similarly in solutions containing high urea concentrations.

The magnitude to which all three proteins are affected is of the same order as that found for serum albumin.[1] This is to be expected as the molecular sizes of these proteins are approximately the same. The radii of the equivalent spheres calculated from the diffusion constants of hemoglobin, β-lactoglobulin and zein [10] are, respectively, 31 Å, 29 Å, and 53 Å compared to 34 Å for serum albumin.[5] The high assymetry of zein gives it a large effective volume despite its low molecular weight of 40 000. This protein may therefore not be directly comparable with the other substances.

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