Studies on Dextran and Dextran Derivatives. X. The Interaction of Dextran Sulfate with Lysozyme*

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ABSTRACT: The interaction between lysozyme and dextran sulfate (M_w 7.6 × 10^3) has been studied under varying experimental conditions. The interaction may lead to the formation of a precipitate containing variable amounts of lysozyme and dextran sulfate. The tendency to precipitate increases at low ionic strength, pH, and temperature. Outside the "precipitation zone" the formation of soluble complexes has been demonstrated by light scattering. A quantitative evaluation of the light-scattering data has been attempted on the basis of models involving a reversible association–dissociation equilibrium and different stoichiometric ratios.

Association–dissociation phenomena between macromolecular components are often encountered in biological systems. Interactions of proteins with other proteins, nucleic acids, and polysaccharides are qualitatively well known, but only in a few cases have been investigated in detail (Nichol *et al.*, 1964).

The system lysozyme[1] and dextran sulfate was chosen in the present work for a quantitative study of the reversible binding of a protein to a dextran derivative. In this case a strong interaction is to be expected on electrostatic grounds: the lysozyme carries a large net positive charge at neutral pH and dextran sulfate has a very high degree of substitution with strongly acidic groups. The system has been studied under several conditions where both soluble and insoluble complexes are formed.

Materials and Methods

Lysozyme. Crystallized lysozyme (muramidase) (M_w 14.5 × 10^3) was obtained from the Worthington Biochemical Corp. Two lots were used (612 and 633). Lysozyme concentrations were determined spectrophotometrically at 280 mμ, using a value of $E^{1\%}_{1cm}$ 25.32 (Bruzzesi *et al.*, 1965).

Dextran Sulfate. The dextran sulfate (obtained from the Societa Italiana Chimici) had a number-average molecular weight (M_n) of 7.0 × 10^3, a weight-average molecular weight (M_w) of 7.6 × 10^3, and a very high degree of substitution (2.3 mequiv/g). Stock solutions were obtained by dissolving weighed amounts of the dried dextran in distilled water or in buffers. After neutralization with 2 N NaOH, the solutions were placed in a boiling water bath for 20 min. The dextran solutions were then clarified by centrifugation at about 15,000 rpm in the multispeed head of an International centrifuge for 1 hr, the slight precipitate being discarded.

Light Scattering. Light-scattering measurements were performed with a Brice Phoenix photometer (Brice *et al.*, 1950) at 546 mμ using narrow slits. The solutions were clarified by filtration through very fine sintered-glass filters directly into a small semioctagonal light-scattering cell. The value of the dissymmetry (τ_{45}/τ_{135}) was very near to unity and never greater than 1.03–1.05 in all the solutions measured. The routine of the measurements was the same as described before (Rossi-Fanelli *et al.*, 1959; Bruzzesi *et al.*, 1965). Unless otherwise stated, the measurements were made at about 20°. No correction for depolarization has been introduced. The value of H in the light-scattering equation

$$Hc/\tau = \frac{1}{M_w} + 2Bc$$

for the lysozyme was taken as 3.92 × 10^{-6} on the basis of a value of dn/dc = 0.1888 ml/g at 546 mμ (Bruzzesi *et al.*, 1965). For dextran sulfate H = 2.61 × 10^{-6} on the basis of dn/dc = 0.154 ml/g at 546 mμ (Antonini *et al.*, 1964).

Ultracentrifugation. Sedimentation experiments were made at 20° in a Spinco Model E analytical ultracentrifuge at 42,040 and 59,780 rpm. The sedimentation coefficients, calculated from the movement of the maximum ordinate, have been adjusted to the viscosity and density of water, and are given in Svedberg units.

Precipitation of Lysozyme by Dextran Sulfate. Precipitation of lysozyme–dextran sulfate mixtures was studied in experiments in which varying amounts of dextran sulfate were added to a solution containing a fixed amount of lysozyme. To a series of centrifuge tubes containing 3 ml of a buffer solution and 3 mg of lysozyme increasing amounts of dextran sulfate were

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[1] Abbreviations used: L, lysozyme; DS, dextran sulfate.

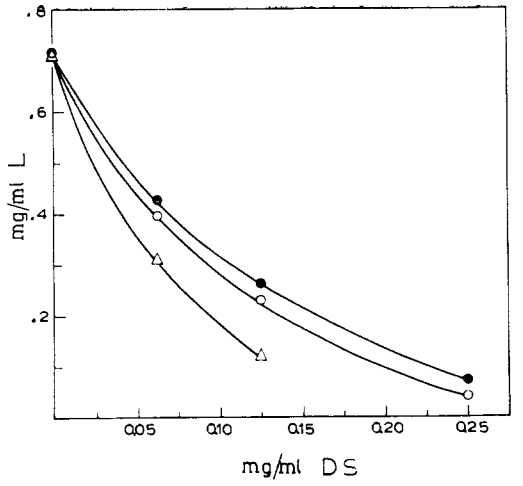

FIGURE 1: Concentration of lysozyme (ordinate) left in solution as a function of dextran sulfate (abscissa) added. ⊙, 0.05 M acetate, pH 4.2; ●, 0.05 M phosphate, pH 7.05; Δ, 0.05 M borate, pH 9.0.

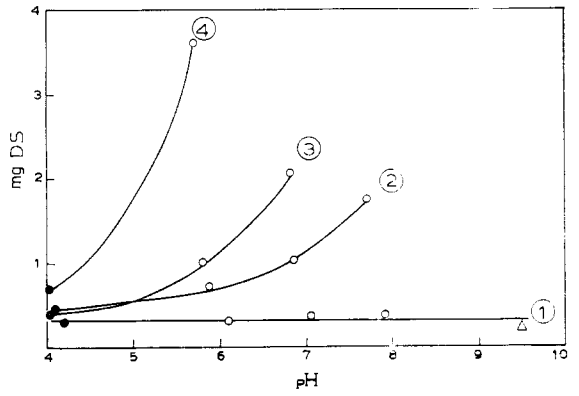

FIGURE 2: Concentration of dextran sulfate precipitating 50% of lysozyme ($c/2$) as a function of pH and ionic strength. Curve 1, 0.05 M buffers; curve 2, 0.05 M buffers + 0.2M NaCl; curve 3, 0.05 M buffers + 0.3 M NaCl; curve 4, 0.05 M buffers + 0.5 M NaCl; ●, acetate; O, phosphate; Δ, borate buffers.

added. The tubes were brought to the same final volume (4 ml) with solvent, and were then left at constant temperature for about 30 min. Afterwards the solutions were centrifuged for 1 hr at about 15,000 rpm at the same constant temperature. At the end of the centrifugation the optical density of the supernatants, *i.e.*, of the lysozyme left in solution, was measured at 280 mμ.

Analysis of the Precipitate. In experiments devised to analyze the precipitate, the precipitation was carried out with much larger amounts of reagents, the other conditions being similar to those described above. The experiments were performed at 0–2°. The precipitate was washed three times with distilled water; the product was lyophilized in tarred centrifuge tubes,

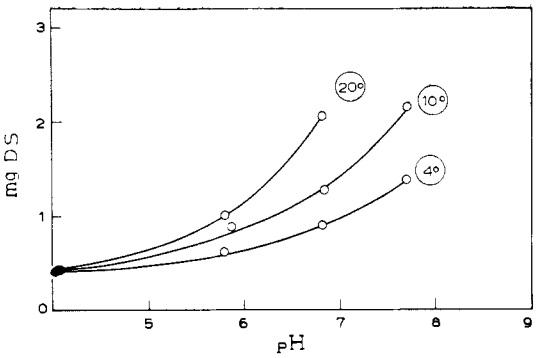

FIGURE 3: Concentration of dextran sulfate precipitating 50% of lysozyme ($c/2$) as a function of pH and temperature. ●, 0.05 M acetate + 0.3 M NaCl; O, 0.05 M phosphate + 0.3 M NaCl.

dried to constant weight, and weighed. The lyophilized material was then solubilized in a known volume of 0.3 M phosphate, pH ~7, and the optical density was measured at 280 mμ. The amount of lysozyme in the precipitate was calculated from the optical density and the amount of dextran sulfate by difference in weight.

Results

Precipitation Experiments. The precipitation of lysozyme has been studied as a function of pH, salt concentration, and temperature.

A. PRECIPITATION AS A FUNCTION OF pH. The concentration of lysozyme remaining in solution, plotted as a function of the dextran sulfate concentration in the pH range 4.2–9.6 in 0.05 M acetate, phosphate, and borate buffers, is shown in Figure 1. The concentration of dextran sulfate corresponding to the precipitation of 50% of the lysozyme initially present ($c/2$) was used to characterize the precipitation curves under different conditions. At very high dextran sulfate concentrations the amount of lysozyme left in solution increases with the amount of dextran sulfate added.

B. PRECIPITATION AS A FUNCTION OF IONIC STRENGTH. The experiments were performed in 0.05 M acetate, phosphate, and borate buffers with added sodium chloride up to a final concentration of 0.2, 0.3, and 0.5 M. In the solutions of pH above 8 no precipitation was obtained at any of the three NaCl concentrations. Figure 2 shows the effect of salt concentration and pH on the precipitation of lysozyme by dextran sulfate: the precipitating effect of dextran sulfate decreases with an increase of ionic strength and pH.

C. PRECIPITATION AS A FUNCTION OF TEMPERATURE. The effect of temperature on the precipitation of lysozyme by dextran sulfate was studied in 0.05 M acetate and phosphate buffers plus 0.3 M NaCl. Figure 3 shows the results obtained at 4, 10, and 20°. The large effect of temperature is particularly striking.

Analysis of the Precipitate. Two sets of precipitates

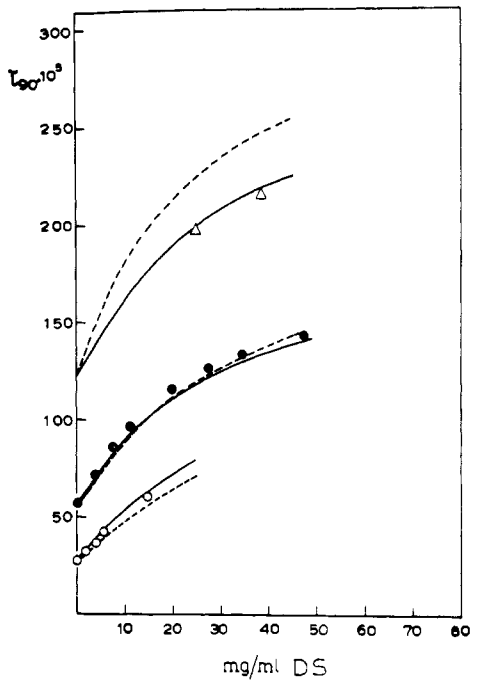

FIGURE 4: Turbidity of dextran sulfate–lysozyme mixtures as a function of dextran sulfate concentration, in 0.3 M phosphate, pH 7. Solid line was calculated for a 1 L/1 DS complex ($K = 2 \times 10^3/M^{-1}$); dashed line for a 2 L/1 DS complex ($K = 3 \times 10^5\ M^{-2}$). ○, lysozyme concentration 4 mg/ml; ●, lysozyme concentration 8 mg/ml; Δ, lysozyme concentration 16 mg/ml.

obtained in different conditions were analyzed; the first was obtained in 0.05 M acetate and 0.5 M NaCl, final pH 4.02, the other in 0.05 M acetate, final pH 4.02. The weight ratio lysozyme/dextran sulfate in the initial mixture varied in both experiments from 1 to 4. The weight ratio lysozyme/dextran sulfate in the precipitate was found to correspond in 0.05 M acetate and 0.5 M NaCl to 1/0.264 and in acetate alone to 1/0.175, and was practically independent of the initial ratio (Table I).

Light-Scattering Experiments. In order to obtain quantitative data on the formation of soluble complexes, the light-scattering properties of the system

TABLE I: Weight Ratio between Lysozyme and Dextran Sulfate.

Buffer (M)	Initial Mixture L/DS	Precipitate L/DS
Acetate (0.05),	1/1.02	1/0.171
pH 4.02	1/2.04	1/0.188
	1/4.09	1/0.167
Acetate (0.05)	1/1.01	1/0.295
+ NaCl (0.5),	1/2.03	1/0.258
pH 4.02	1/4.06	1/0.240

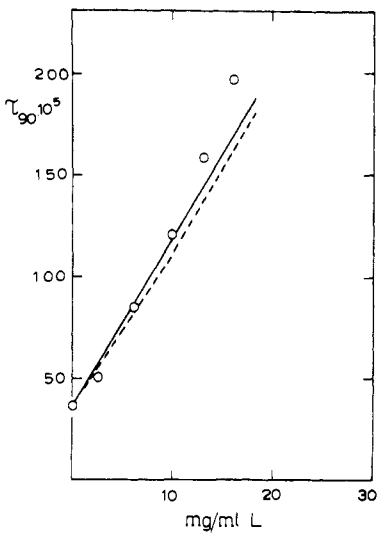

FIGURE 5: Turbidity of a dextran sulfate–lysozyme mixture as a function of lysozyme concentration, 0.3 M phosphate, pH 7, 20°. Solid line was calculated for a 1 L/1 DS complex ($K = 2 \times 10^3\ M^{-1}$); dashed line for a 2 L/1 DS complex ($K = 3 \times 10^5\ M^{-2}$). Dextran sulfate concentration, 25 mg/ml.

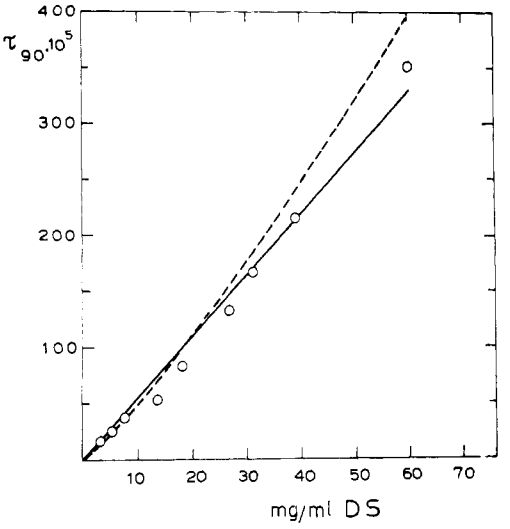

FIGURE 6: Turbidity of a mixture containing dextran sulfate and lysozyme at a fixed ratio as a function of dextran sulfate concentration. Initial concentration: dextran sulfate 60 mg/ml, lysozyme 24.3 mg/ml, in 0.3 M phosphate, pH 7. Solid line was calculated for a 1 L/1 DS complex ($K = 2 \times 10^3\ M^{-1}$); dashed line for a 2 L/1 DS complex ($K = 3 \times 10^5\ M^{-1}$).

lysozyme–dextran sulfate have been investigated under conditions where no precipitation occurs, *i.e.*, in 0.3 M phosphate buffer, pH 7, and ~20°.

The light scattering of the two isolated components of the system was first studied in detail as a function

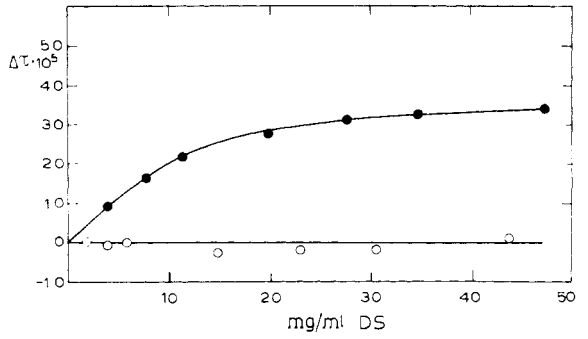

FIGURE 7: $\Delta\tau$ (difference between the scattering of dextran sulfate–lysozyme mixtures and that of the isolated components) as a function of dextran sulfate concentration. ●, 0.3 M phosphate, pH 7 (lysozyme concentration 10 mg/ml); ○, 0.15 M phosphate, pH 7, + 1 M NaCl (lysozyme concentration 5 mg/ml).

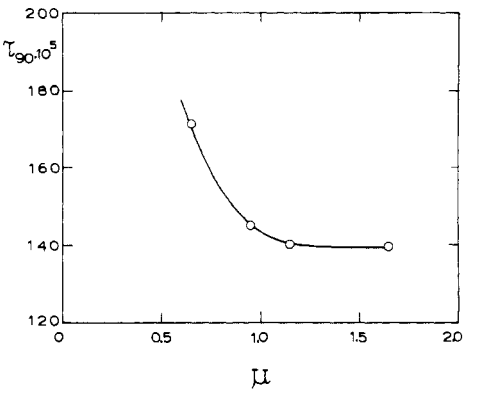

FIGURE 9: Turbidity of a lysozyme–dextran sulfate mixture as a function of ionic strength. Lysozyme concentration 12.44 mg/ml, dextran sulfate concentration 30.7 mg/ml, in 0.3 M phosphate, pH 7, plus NaCl.

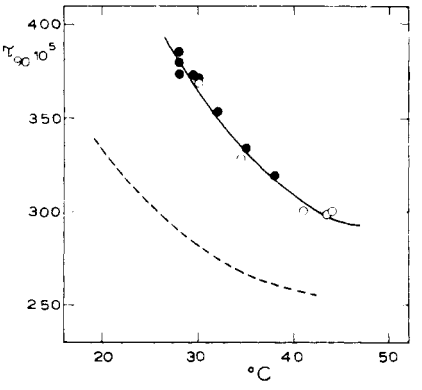

FIGURE 8: Turbidity of a lysozyme–dextran sulfate mixture as a function of temperature. Lysozyme concentration 36 mg/ml, dextran sulfate 18 mg/ml, in 0.3 M phosphate, pH 7. Dashed line shows the effect of temperature on a 36-mg/ml lysozyme solution (Bruzzesi *et al.*, 1965). ●, decreasing temperatures; ○, increasing temperatures.

of concentration. The results can be fitted by the following equations (obtained by a least-squares procedure): lysozyme $\tau_{90} \times 10^2 = 6.5557c + 0.079508c^2$; dextran sulfate $\tau_{90} \times 10^2 = 1.8276c - 0.014878c^2$. The weight-average molecular weight of dextran sulfate was calculated to be 7.6×10^3; hence the ratio M_w/M_n is very near to unity.

Light-scattering measurements were then performed on the following systems: (a) solutions containing a constant amount of lysozyme and different amounts of dextran sulfate (Figure 4); (b) solutions containing a fixed amount of dextran sulfate and variable amounts of lysozyme (Figure 5); (c) solutions containing a fixed ratio of dextran sulfate to lysozyme, but at different absolute concentrations (Figure 6).

The results of these experiments indicate that formation of a soluble complex between dextran sulfate and lysozyme takes place under the conditions of the experiments. The complex is revealed in a plot of $\Delta\tau$ (the difference between the scattering of the dextran sulfate–lysozyme solution and the sum of the turbidities of the isolated components at the same concentration as in the mixture, assuming no interaction) *vs.* dextran sulfate concentration (Figure 7). Figure 7 also shows that adding 1 M NaCl abolishes the complex formation; the turbidity of the lysozyme–dextran sulfate system becomes equal to the sum of the turbidities of the isolated components. The effect of temperature and ionic strength have been studied with solutions containing a fixed ratio of lysozyme to dextran sulfate; the results are reported in Figures 8 and 9.

Analysis of the Light-Scattering Data. The method used was to consider that a reversible association–dissociation equilibrium was present. The properties of the complex were calculated for different models, *i.e.*, complexes formed by association of dextran sulfate and lysozyme monomer in the molecular ratios 1/1, 1/2, and 2/1; the quantitative evaluation rests upon the following simplifying assumptions. (a) Only one type of complex, with a weight-average molecular weight (M_w) equal to the sum of the M_w of the components, is present; (b) lysozyme and dextran sulfate contribute to the dn/dc of the complex according to their weight ratios in the complex; (c) B, the second virial coefficient, is equal to 0 for the complex; (d) dextran sulfate is monodisperse; (e) the turbidity (τ) of the solution containing free lysozyme, free dextran sulfate, and complex is equal to the sum of the turbidities of the components; (f) the light-scattering equation in its simple form

$$\frac{Hc}{\tau} = \frac{1}{M_w} + 2Bc$$

may be used.[2]

According to these assumptions standard turbidity curves for the three kinds of complexes have been calculated. Turbidity *vs.* concentration curves for lysozyme and dextran sulfate were obtained experimentally as described above. The mass law was applied to each and the concentrations of free lysozyme, dextran sulfate, and complex calculated with different values of the equilibrium constants. The value of τ was then obtained from the sum of the τ values of each component. The calculated τ values were compared with the experimental ones until a satisfactory fit was obtained.

A comparison between calculated and experimental values is shown in Figure 4. Of the three models tried, the one corresponding to 1 lysozyme + 2 dextran sulfate $\rightleftharpoons$ complex might be excluded since the theoretical turbidity curves *vs.* dextran sulfate concentration have a sigmoidal shape which is different from the experimental one. The two models 1 lysozyme + 1 dextran sulfate $\rightleftharpoons$ complex and 2 lysozyme + 1 dextran sulfate $\rightleftharpoons$ complex give a similar satisfactory fit at low lysozyme concentrations, but the 1/1 model seems to fit the data at high lysozyme concentrations better. The equilibrium constants used for these fits are 2×10^3 M^{-1} and 3×10^5 M^{-2}, respectively. The fits, obtained with these equilibrium constants for the data at a fixed dextran sulfate concentration and variable amounts of lysozyme, are shown in Figure 5; the fits for data obtained by diluting solutions of dextran sulfate + lysozyme in a fixed ratio are shown in Figure 6; the agreement between the experimental and the calculated values is not as good as for the data of Figure 4.

Sedimentation Experiments. The sedimentation velocity of lysozyme, dextran sulfate, and mixtures of the two was measured in 0.3 M phosphate buffer, pH 7, at 20°. The results are shown in Table II.

TABLE II: Sedimentation Coefficients of Lysozyme, Dextran Sulfate, and Their Mixture.[a]

Components	Concn (mg/ml)	$s_{20,w}$
Lysozyme	14.5	2.01
Dextran sulfate	7.6	1.20
Lysozyme + dextran sulfate	14.5 + 7.6	1.93
Lysozyme + dextran sulfate	8.28 + 4.34	1.74
Lysozyme + dextran sulfate	6.44 + 3.38	1.71

[a] Phosphate (0.3 M) buffer, pH 7, 20°.

The lysozyme–dextran sulfate mixtures show only one broad peak with sedimentation rates between those of the isolated components. While these results are not inconsistent with the formation of a soluble complex, they do not offer direct evidence for its existence.

Discussion

The system lysozyme–dextran sulfate provides a suitable model for the interaction between charged macromolecules. As shown above, the interaction leads to the formation of both insoluble and soluble complexes depending on pH, salt concentration, and temperature. Both phenomena appear to be fully reversible on changing the experimental conditions. This is shown for instance by the effect of temperature reported in Figure 8.

The electrostatic nature of the interaction between lysozyme and dextran sulfate is reflected in the effect of pH and especially of ionic strength. The behavior of the system is therefore similar to other systems recently described in the literature, in which electrostatic interactions dominate, *i.e.*, polyamino acid and proteins (Rice *et al.*, 1954; Sela and Steiner, 1963), proteins and proteins (Steiner, 1953), polysaccharides and proteins (Schubert and Franklin, 1961; Tsang and Thompson, 1965), and antigen–antibody (Katchalski, 1962; Pepe and Singer, 1959). These systems all behave in a qualitatively similar way: the insoluble complexes form at low ionic strength in pH ranges which vary from system to system. Outside the precipitation zone the precipitates dissolve and soluble complexes may form. The weight ratio of the components in the precipitate is independent of their initial ratio, but varies according to the experimental conditions (Tsang and Thompson, 1965).

In the present work the formation of the soluble complex is shown unequivocally by the light-scattering data, and a quantitative treatment to establish the stoichiometry and equilibrium constant of the reaction has been attempted. The treatment might appear oversimplified, not only because of the numerous assumptions involved, but also because the effect of the polymerization of lysozyme (Sophianopoulos and Van Holde, 1964; Bruzzesi *et al.*, 1965) on the equilibrium between the protein and dextran sulfate has been neglected. However, most of the assumptions seem to be justified, at least as a first approximation, and a great number of measurements were done at a lysozyme concentration below 1%, where the polymerization of lysozyme is small (Bruzzesi *et al.*, 1965). Actually the fit between the calculated and observed values of τ is good using the model involving a 1/1 association between dextran sulfate and lysozyme, especially for the data obtained at low

[2] The use of this light-scattering equation implies that the system is a two-component one, consisting of macromolecules and solvent, and neglects any selective interaction between the "nondiffusible" components and any of the "diffusible" ones. However these assumptions seem to be justified, at least as a first approximation, in view of the fact that Hc/τ, extrapolated to zero concentration, gives correct values of $1/M_w$ both for lysozyme and dextran sulfate (Casassa and Eisenberg, 1964).

lysozyme concentration. It might be pointed out that a better description of the system could be achieved by considering the occurrence of multiple equilibria of the type:

$$\text{lysozyme} + \text{dextran sulfate} \rightleftharpoons \text{complex}_1$$

$$\text{complex}_1 + \text{lysozyme} \rightleftharpoons \text{complex}_2$$

However, solutions for such models are not easily obtained by desk calculations, and therefore have not been attempted here. The value of the equilibrium constant for this model lies in the same range as those reported for other similar systems (Steiner, 1953; Tsang and Thompson, 1965).

It is more difficult to account for the effects of ionic strength and temperature in terms of values of the equilibrium constant, especially since no extensive data are available. The data shown in Figures 8 and 9 suggest that the equilibrium constant (1/1 model) decreases about 20 times for addition of NaCl up to 1 M to the 0.3 M phosphate buffer, and that the ΔH for the association is of the order of 50 kcal mole^{-1}.

A quantitative evaluation of the sedimentation results has not been attempted. Preliminary considerations showed that sedimentation methods were not particularly suited to the detection of the formation of soluble complexes of lysozyme and dextran sulfate. This agrees with the conclusions reached by other authors on similar systems (Schubert and Franklin, 1961). However, calculations based on the stoichiometry and equilibrium constant deduced from the light-scattering experiments indicated that values of sedimentation coefficients intermediates between those of the two compounds were to be expected under the conditions employed.

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