Figure 9 gives examples of such calculations, useful for enzymological applications.

In summary, titration curves for EDTA, ATP, and PP in the presence of alkali metal ion, M, have been analyzed in terms of a scheme which allows for binding of metal ions to give the species HMY^{2-}, MY^{3-}, and M_2Y^{2-}, where Y represents the compound being titrated. The apparent stability constants for metal binding are tabulated and can be used to calculate the fraction of Y in each of its various forms as a function of [M] and *p*H.

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Association-Dissociation Properties of Lysozyme*

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ABSTRACT: The association-dissociation properties of lysozyme have been studied by light scattering and sedimentation velocity. The protein monomer associates at *p*H > 4.5; the data indicate that association-dissociation equilibria involving higher polymers than dimers become important at high protein concentrations.

In the course of a study on the interaction of dextran sulfate with lysozyme (muramidase), evidence was obtained by light-scattering measurements that lysozyme associates reversibly under certain experimental conditions. This observation is in line with the recently published studies of Sophianopoulos and Van Holde (1964) which indicate that lysozyme monomer undergoes a *p*H-dependent reversible association into dimers. Results of light-scattering and ultracentrifugation experiments are reported here, which indicate that the reversible association of lysozyme goes beyond the dimer stage.

Materials and Methods

Lysozyme. Crystallized lysozyme (muramidase) was obtained from the Worthington Biochemical Corp. Two lots were used (612 and 633). Lysozyme concentrations were determined by optical density measurements at 280 mμ, using a value of $E^{1\%}_{1\,cm} = 25.32$ (value determined in this laboratory by Dr. U. Ferrini with dry weight measurements on lot 612).

Ultracentrifugation. Sedimentation coefficients were measured at 20° in a Spinco Model E analytical ultracentrifuge at 59,780 rpm. The values of the sedimentation coefficients have been adjusted to the viscosity and density of water and are given in Svedberg units.

Light Scattering. Light-scattering measurements were performed with a Brice Phoenix photometer (Brice *et al.*, 1950) at 546 mμ. The solutions were clarified by filtration through very fine sintered-glass filters directly into the light-scattering cell; the routine of the measurements was the same as described before (Rossi Fanelli *et al.*, 1959).

In most cases small semioctagonal cells were used. 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 value of H in the light-scattering equation ($Hc/\tau = 1/M_w + 2Bc$) was taken as 3.92×10^{-6} on the basis of a value of $dn/dc = 0.1888$ ml/g at 546 mμ. This value was obtained by measurements of dn with a differential refractometer using the specific extinction coefficient reported here. It agrees with the value of dn/dc reported in the literature (Stacey, 1956).

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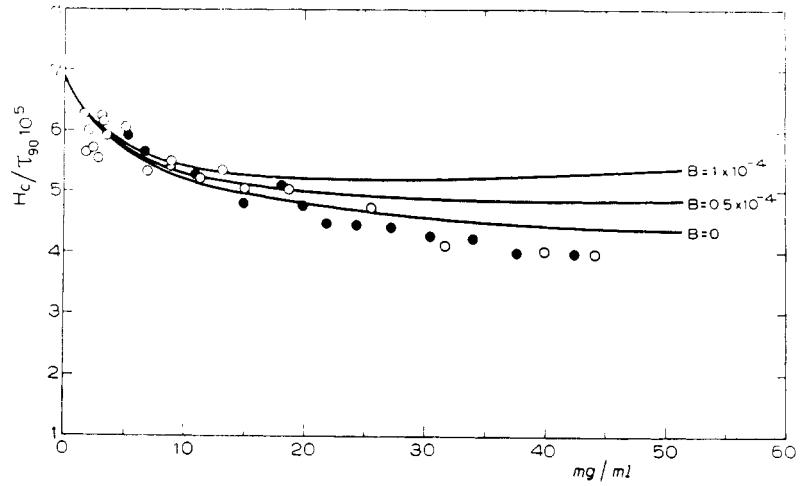

FIGURE 1: Light-scattering data for lysozyme at *p*H 6.8 in 0.3 M phosphate buffer. Solid lines were calculated for a monomer-dimer equilibrium ($K = 5 \times 10^2$ M^{-1}) with the values of B shown in the figure.

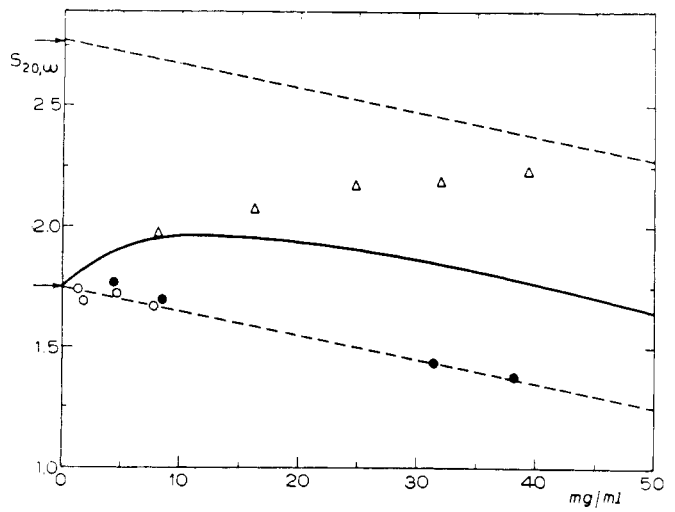

FIGURE 2: Concentration dependence of $s_{20,w}$ of lysozyme at various *p*H values. Dashed lines represent the calculated concentration dependence of monomer and dimer (see text). Solid line was calculated for a monomer-dimer equilibrium ($K = 5 \times 10^2$ M^{-1}). △, 0.3 M phosphate buffer, *p*H 6.8; ○, 0.3 M glycine-HCl buffer, *p*H 3.2; ●, 0.4 M Na acetate buffer, *p*H 4.3.

Results

Light Scattering and Sedimentation of Lysozyme at Neutral pH in Phosphate Buffer. The results of light-scattering measurements on lysozyme solutions at *p*H 6.8–7.0 in 0.3 M potassium phosphate buffer at temperatures near 20° are shown in Figure 1. In these experiments the most concentrated solution was measured first and the points at lower concentrations were obtained by dilution of this solution with solvent. After each dilution the solution was filtered again; the concentration was determined on samples collected after each light-scattering measurement. The light-scattering readings were obtained within a minute or so after addition of the solvent and no change was observed with time up to several hours.

The concave shape of the light-scattering curves shown in Figure 1 indicates that lysozyme behaves as an associating-dissociating system. The values of Hc/τ extrapolated to zero protein concentration give a value of $M_w = 14.5 \times 10^3 \pm 5\%$ which is in agreement with the chemical molecular weight of the protein (Canfield, 1963). Essentially similar results were obtained also at *p*H 8.2 in 0.3 M K_2HPO_4. The two different lots of lysozyme behaved identically.

The sedimentation coefficients of lysozyme, under conditions similar to those of the light-scattering meas-

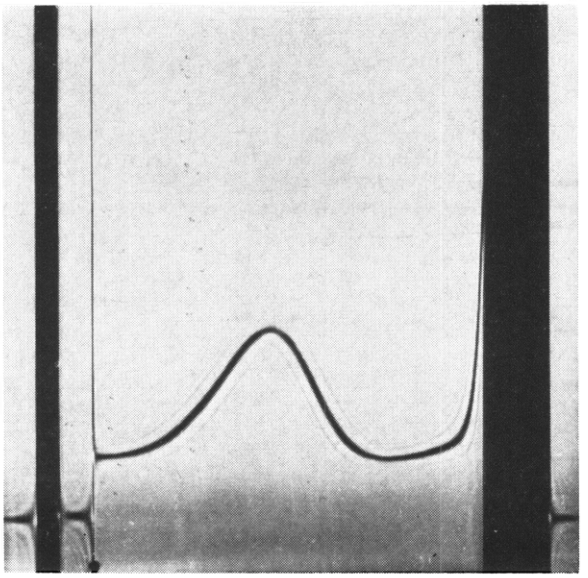

FIGURE 3: Ultracentrifuge pattern of lysozyme. Protein concentration 44.3 mg/ml in 0.3 M phosphate buffer, *p*H 6.8. Picture taken 206 minutes after the ultracentrifuge reached full speed of 59,780 rpm.

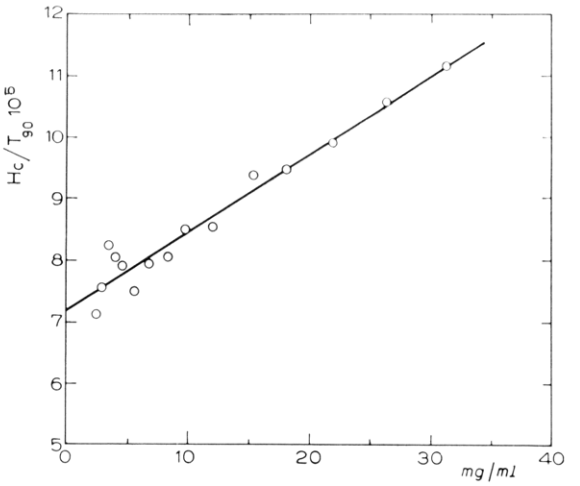

FIGURE 4: Light-scattering data for lysozyme at *p*H 2.2 in 0.3 M glycine-HCl buffer.

urements just described, are reported in Figure 2. The sedimentation pattern showed in all cases a single, fairly symmetrical peak (Figure 3). The sedimentation results too indicate that the lysozyme monomer associates to form higher molecular weight species with increasing protein concentration. The value of $s_{20,w}$ for the monomer obtained from extrapolation to zero protein concentration leads to a value of f/f_0 of 1.2, which is consistent with the behavior of globular proteins in solution (Tanford, 1961).

Light Scattering and Sedimentation of Lysozyme in Acid Solutions. Light-scattering data on lysozyme solutions at acid *p*H are shown in Figure 4. It is evident that the value of Hc/τ extrapolated to zero protein concentration is very similar to that obtained in the neutral *p*H range. On the other hand, the data indicate absence of association at finite protein concentrations. The light-scattering plots have a positive slope which is "normal" for nonassociating protein systems at moderate ionic strength and high charge (Tanford, 1961). The value of B (the virial coefficient term in the light-scattering equation $Hc/\tau = 1/M_w + 2Bc$), which can be calculated from the slope at *p*H 2.2, agrees with that found for other proteins under similar conditions (Edsall *et al.*, 1950; Tanford, 1961). Values of sedimentation coefficients of lysozyme in acid solutions are shown in Figure 2. Again, in agreement with the light-scattering data, the "normal" concentration dependence of s indicates the absence of association. The value of $s_{20,w} = 1.75$ S corresponds to that of the lysozyme monomer. In acid solutions, as in neutral ones, no change with time of the light scattering and of the sedimentation was observed over a period of hours.

Effect of pH on Light Scattering of Lysozyme. In these experiments a concentrated solution of lysozyme (about 5%) in 0.3 M NaCl was titrated with NaOH or HCl and the light scattering was measured at the different *p*H values so obtained. The data reported in Figure 5 are consistent with the idea that lysozyme undergoes a *p*H-dependent association-dissociation equilibrium in the *p*H range 4.5–6.5, as also indicated by the data of Sophianopoulos and Van Holde (1964). It should be pointed out that the light-scattering readings, which were made as soon as possible after changing the *p*H of the solutions, remained constant, and also that for any given *p*H the value of τ was the same irrespective of the initial *p*H of the solution.

Effect of Temperature on Light Scattering of Lysozyme. The light-scattering cell which contained the solutions of lysozyme was immersed in a water bath at different temperatures. After the solution had reached a given temperature, which could be read from a thermometer immersed in the cell, the cell was quickly placed in the light-scattering photometer and the readings were taken; afterwards the temperature was measured again. This procedure ensured that no significant change in temperature occurred within the time employed for the light-scattering readings. The results of measurements of this kind on solutions of lysozyme at *p*H 6.8 and 2.2 are shown in Figure 6.

The data at *p*H 2.2 and about 4% protein concentration show only a very small change in τ with change in temperature. It is of the same size as that found for other nonassociating proteins, e.g., serum albumin, and reflects changes in the light-scattering constant (H) with temperature (Tanford, 1961).

On the other hand, at *p*H 6.8 lysozyme solutions show a marked change in τ with temperature, which appears to be due to the effect of changed temperature on its association equilibrium. The data also show that the value of τ is independent of the side from which the final temperature is approached.

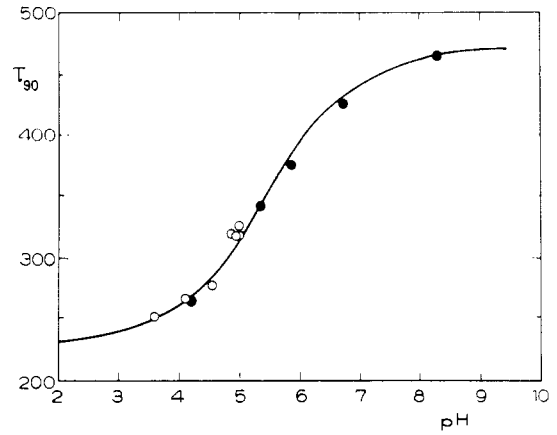

FIGURE 5: Turbidity (τ_{90}) of lysozyme at different *p*H values. Protein concentration 48 mg/ml in 0.3 M NaCl titrated with NaOH (●) or HCl (○).

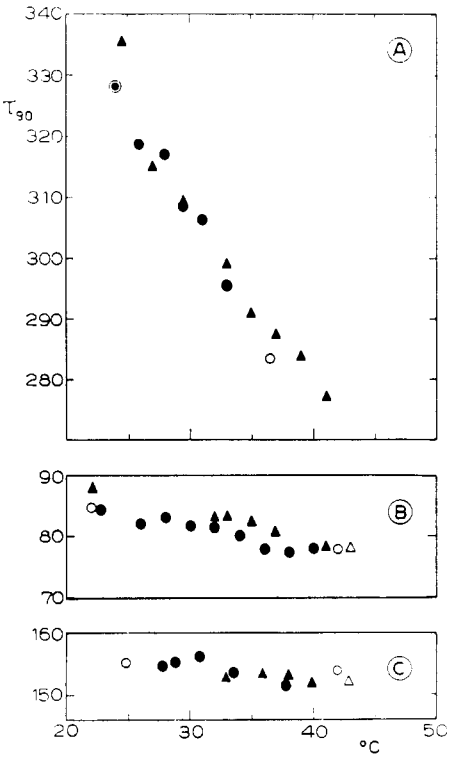

FIGURE 6: Effect of temperature on turbidity (τ_{90}) of lysozyme at different *p*H values. ○, △, increasing temperatures; ●, ▲, decreasing temperatures. (A) Protein concentration 38 mg/ml in 0.3 M phosphate buffer at *p*H 6.8; (B) protein concentration 14 mg/ml in 0.3 M phosphate buffer at *p*H 6.8; (C) protein concentration 45 mg/ml in 0.3 M glycine-HCl buffer at *p*H 2.2.

Discussion

The light-scattering and sedimentation results reported here show that the lysozyme monomer undergoes a *p*H-dependent association, in agreement with the conclusions of Sophianopoulos and Van Holde (1964). The data also indicate that we are dealing with a true, reversible, rapidly established association-dissociation equilibrium between the monomer and higher polymers. These conclusions are based upon the reversibility and the rapid attainment of the final values of the light-scattering measurements as well as upon the companion ultracentrifuge experiments in which the sedimentation patterns showed, under all conditions, only one rather symmetrical peak.

A quantitative treatment of the light-scattering and sedimentation data requires the correlation of the measured quantities (Hc/τ and s) with the weight-average molecular weight. For the light-scattering data the equation $Hc/\tau = 1/M_w + 2Bc$ can be used. If this equation is used, it is assumed that the system is a two-component one, consisting of macromolecules and solvent, and that there is no interaction between the high molecular weight components and any of the diffusible ones. These assumptions are justified to a large extent for proteins dissolved in salt solutions of moderately high ionic strength (Casassa and Eisenberg, 1964). In the present case the validity of the assumption is also justified by the fact that the value of Hc/τ extrapolated to zero protein concentration gives a value of $1/M_w$, which corresponds to the chemical molecular weight of the lysozyme monomer.

The evaluation of the term Bc in the previous equation represents another difficulty. In protein solutions at $\mu > 0.05$, B has a small positive value, even if the protein carries a high net charge. The term $2Bc$ can usually be neglected at protein concentrations below 1%. The measurements, however, were extended at much higher protein concentrations and the contribution of $2Bc$ becomes relevant for the determination of M_w. The value of B of the lysozyme monomer can be obtained directly from the data at *p*H 2.2 where the association should be negligible; under any other condition a rough estimation of its size can be made from that of B at *p*H 2.2 and the known dependence of B on the charge of the protein and on the ionic strength (Edsall *et al.*, 1950; Tanford, 1961). In the case of associating proteins it has been shown that B is the same for the associated and dissociated proteins (Timasheff and Kronman, 1959).

The value of B at *p*H 2.2, where lysozyme carries a net charge of about 18 per mole of protein, has been calculated from the data of Figure 4 to be about 6×10^{-4}. At *p*H 7, where the net charge is 7 and at $\mu = 0.6$, the value of B may be estimated to lie near to 1×10^{-4}.

Having assigned an order of magnitude to B, an analysis of the light-scattering data at *p*H 6.8 shown in Figure 1 can be attempted. The curves have been calculated for a monomer-dimer equilibrium assuming a value of $K = 5 \times 10^2$ M^{-1} and using different values for B (0–1×10^{-4}) from the following equation:

$$K = \frac{\left(\frac{M_2}{M_1} - 1\right)}{2c\left(2 - \frac{M_2}{M_1}\right)^2}$$

where M_1 and M_2 are the molecular weights of monomer and dimer, respectively, and c is the concentration in mg/ml. At low protein concentrations ($c < 2\%$) the model fits the experimental data; however for protein concentrations greater than 2% there is a large departure of the calculated values of Hc/τ from those obtained experimentally, even if a value for B of zero is assumed.[1] The data indicate that the association of lysozyme at *p*H 6.8 and moderate ionic strength goes beyond the dimer stage. A single equilibrium of the form n Ly $\rightleftharpoons$ Ly$_n$, with $n > 2$, also seems inadequate to describe the experimental data, the curves of Hc/τ versus c having in this case, especially at low protein concentrations, a different shape from those of the experiments shown in Figure 1. The data reported here suggest that, while at low protein concentrations the predominant equilibrium is

$$\text{monomer} \rightleftharpoons \text{dimer}$$

at high protein concentrations higher molecular weight species are formed in appreciable amount.

The sedimentation data at *p*H 6.8, in agreement with the light-scattering measurements, also indicate that the association of lysozyme is not a simple monomer-dimer equilibrium. Theoretical weight-average velocities as a function of concentration have been calculated for a monomer-dimer equilibrium, using $K = 5 \times 10^2$ M^{-1} again. The monomer and dimer particles have been treated as spheres so that $s_2 = 1.5845 s_1$. The velocity of the dimer has been regarded as decreasing linearly with concentration with the same concentration dependence obtained for the monomer at acid *p*H values; thus $s_1 = 1.75(1 - 0.06\,c)$ and $s_2 = 2.78(1 - 0.06\,c)$.

A comparison of the calculated curves and the experimental data is shown in Figure 2. A model consisting of a single monomer-dimer equilibrium can be applied only to the data at concentrations lower than 2% as for the light-scattering. At higher concentrations the association is much more pronounced than that calculated according to the model; this suggests the presence of a further association of the dimer. The presence of a single peak is consistent with the exclusion of a single equilibrium between monomers and forms higher than the dimers, which also is suggested by the light-scattering data. According to Gilbert (1959) in this case a single peak should not be observed; this theory, however, does not take hydrodynamic effects into account.

The light-scattering and sedimentation data on lysozyme at neutral *p*H and high protein concentration could be treated in principle in terms of progressive association involving several equilibrium constants, each one corresponding to a stage of the association. Such an analysis has been carried out using the method described by Steiner (1952). It gave for the initial monomer-dimer stage a value of $K \sim 5 \times 10^2$ M^{-1} and excluded single equilibria between monomers and forms higher than the dimers. The analysis of the successive stages of association, however, appeared to be limited by the uncertainties on the values of B which prevent an accurate evaluation of M_w at high protein concentration.

The impossibility of describing the association of lysozyme in terms of a single equilibrium constant does not permit an exact evaluation of the effect of *p*H and temperature on the association of lysozyme. The data indicate however that the protein is essentially all monomeric even at concentrations near 5% below *p*H 4.5, and that an increase in temperature at *p*H 7 promotes dissociation of the higher molecular weight forms.

[1] Under the conditions used in these experiments negative values of B may be excluded (Edsall *et al.*, 1950; Tanford 1961).

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