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DIFFUSIVITY OF PROTEIN IN AQUEOUS SOLUTIONS

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Abstract – The diffusion coefficient of lysozyme, a globular protein, was measured at various conditions as functions of lysozyme concentration, salt concentration, and solution 'age' in concentrated, saturated, and supersaturated solutions, employing Gouy interferometry. Distilled water, 0.05 M potassium phosphate buffer, and 0.1 M sodium acetate buffer solutions with 0, 2, 4, and 5 wt% NaCl were used as solvents. The pH of lysozyme solutions in distilled water was 4.75 due to the self-buffering capacity of lysozyme. The pH's of the lysozyme solutions in the potassium phosphate and sodium acetate buffers were adjusted to 6.8 and 4.0, respectively. The experimental temperature was 25°C. In a salt-free system, the concentration dependent diffusion of lysozyme showed typical electrolyte diffusion behavior, while a salt-polyelectrolyte system exhibited the behavior of a non-electrolyte. Diffusion results in the supersaturated region showed a little effect of concentration or solution 'age' at a fixed NaCl concentration. A rapid decline in diffusion coefficient with increasing NaCl concentration in the supersaturated region, however, was observed.

Key words: Lysozyme, Diffusivity, Salt, Supersaturation, Interferometer

INTRODUCTION

Crystallization is an important separation and purification technique for a wide range of materials. For crystallization to occur, the solution must be supersaturated. For that reason, the properties of supersaturated solutions and their relation to crystal growth and nucleation processes are of interest.

Myerson and coworkers studied the diffusion of a number of materials (urea, glycine, NaCl, KCl) in supersaturated solutions employing Gouy interferometry [Chang, 1985; Chang and Myerson, 1985, 1986; Lo, 1991; Myerson and Lo, 1991; Myerson and Senol, 1984; Sorell, 1981]. The diffusion coefficients in all cases declined sharply with increasing concentration in the supersaturated region. In addition, the diffusion coefficient was also found to be a weak function of solution 'age', declining slowly as time increased. It has been postulated that the reason for the observed decline in diffusion coefficient in supersaturated solutions is a result of molecular clusters or aggregates evolving in the supersaturated solutions [Lo, 1991; Myerson and Lo, 1991].

The growth of protein crystals is of great interest for the determination of protein crystal structure. In order to understand the crystal growth of proteins information about their diffusion coefficients and cluster formation at a variety of conditions is of importance. It is the purpose of this work to examine the diffusion coefficient of lysozyme, a globular protein, in concentrated, saturated, and supersaturated solutions with various buffers and salt concentrations.

BACKGROUND

Diffusion of neutral macromolecules in dilute solution is well described by an expression which employs the frictional coefficient of the molecule, such as the Stokes-Einstein equation. However for biological macromolecules, such a simple equation cannot be employed, because they show a strongly non-ideal behavior, even in dilute solutions [Daniel and Alexandrowicz, 1963; Doherty and Benedek, 1974; Ermak, 1974; Kedem and Katchalsky, 1955; Lee and Schurr, 1975; Raj and Flygare, 1974; Tivant, 1983; Varoqui and Schmitt, 1972]. Recently, a number of studies investigated diffusion of biological macromolecules. These studies indicate that the diffusion coefficient of these molecules depends not only on the friction factor, but also their charge, concentration and the ionic strength of the solution [Daniel and Alexandrowicz, 1963; Doherty and Benedek, 1974; Ermak, 1974; Kedem and Katchalsky, 1955; Lee and Schurr, 1975; Raj and Flygare, 1974; Tivant, 1983; Varoqui and Schmitt, 1972].

When protein molecules dissolve in water, the molecules ionize into macroions and small counterions. When the molecules undergo translational and rotational movements, differences in ionic mobilities between macroions and counterions are created in the solution due to their sizes. Ionized molecules (macroions and counterions) then cause a gradient of electrical potential due to the differences of ionic mobilities [Vink, 1990]. Macroions interact very strongly with the solvent molecules (usually buffer) and with other macromolecules because of the electrostatic forces between them. This effect is important in looking at the diffusion coefficients of biological macromolecules. The diffusion of macroions, thus, depends on both electrical and hydrodynamic properties of all ionic components. The size and shape of the macroions may depend on the charge and the interaction with counterions. It is also known that the ionic strength of the solution might affect the hydrodynamic factor.

It has been shown that the role of the frictional coefficient on the solution properties of the biological macromolecules is

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minor, compared to the charge effect [Alexandrowicz, 1963]. The charge on the macromolecule may be shielded by an atmosphere of counterions, or sometimes salt components in the solution, depending on the environmental conditions. It is necessary for the ionic atmosphere to move with the macromolecule. When there is no salt in the solution even at infinite dilution, the ionic atmosphere surrounding the charged macromolecule is not suppressed and consequently the charge effect of protein does not vanish. Because the macroions and the counterions are coupled in the absence of salt [Varoqui and Schmitt, 1972; Vink, 1990], they act like electrolytes. The electrical effect of the highly charged macroion is expected to be screened when small ions are introduced, e.g., by the addition of simple electrolytes [Varoqui and Schmitt, 1972; Vink, 1990]. The molecular charges on the macroion can cause intramolecular repulsion, thus leading to molecular expansion. Added salt changes the ionic strength of the solution without altering the charge on the protein. With increasing ionic strength of the solution the salt component can shield this charge effect, and the effect of the molecular expansion is reduced. Electrostatic interaction thus plays a crucial role in the diffusion of macroions. At high salt concentrations, the charge effect is diminished, meaning that high ionic strength suppresses the effect of the charge on the hydrodynamic properties of the macromolecule [Kedem and Katchalsky, 1955; Varoqui and Schmitt, 1972; Vink, 1990].

EXPERIMENTAL

1. Materials

Chicken egg white lysozyme (grade IV) chlorice purchased from Sigma Chemical Co. was used for this work. Distilled water, 0.05 M potassium phosphate buffer and 0.1 M sodium acetate buffer solutions with 0, 2, 4, and 5 wt% NaCl were prepared as solvents for lysozyme. NaCl was used as a precipitating agent because it is known that NaCl has little effect on conformational change of lysozyme with its concentration, which means NaCl does not denature the protein.

The pH values of each buffer solution were adjusted to 6.8 for 0.05 M potassium phosphate buffer and to 4.0 for 0.1 M sodium acetate buffer solutions by adding concentrated HCl or NaOH. For distilled water, the pH value of the lysozyme solution was constant (4.75), even if a small amount of lysozyme was dissolved in the water because of the self-buffering capacity of lysozyme.

2. Solubility

Solubilities of lysozyme at 25°C in various solutions (distilled water, 0.05 M potassium phosphate buffer solution, and 0.1 M sodium acetate buffer solutions with various amounts of NaCl, i.e., 0, 2, 4, and 5 wt%) were measured using a spectrophotometer (Cary 2300 by Varian). Saturated solution with excess solute was filtered by 0.2 μm filter. The absorbance of the filtered solution, then, was measured by UV spectrophotometer. The saturation concentration was calculated by the following equation.

$$A = \varepsilon C l \tag{1}$$

where A is the absorbance, ε the molar absorption coefficient, l the sample cell thickness, and C the sample concentration.

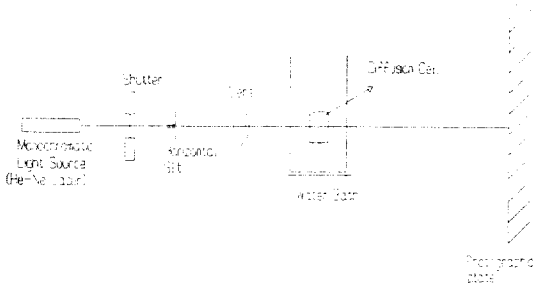


Fig. 1. Schematic diagram of the Gouy interferometer.

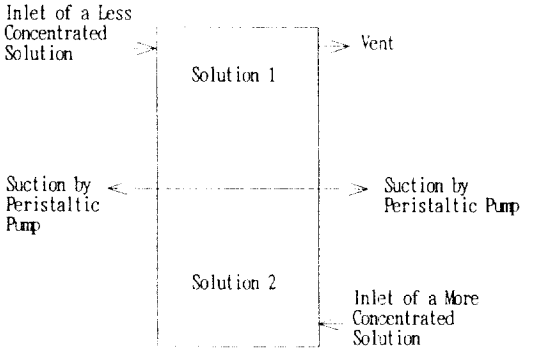


Fig. 2. Diffusion cell.

The absorbance of 1 wt% lysozyme solution in a 1 cm cell at 280 nm was presumed to be 27 [Elgersma et al., 1992].

3. Diffusion Coefficient

The diffusion coefficients of lysozyme in various buffer solutions at 25°C were measured as functions of lysozyme concentration, salt concentration, and solution age employing a Gouy interferometer. A schematic diagram of the interferometer appears in Fig. 1. Illumination of the system was provided by a Spectra Physics (Model 146) randomly polarized helium neon laser with an output power of 4 mW and an output wave length of 633 nm. A more detailed description of the apparatus can be found in Sorrell [1981] and Chang [1985]. A single plexiglass constant temperature bath was used to control the temperature of the solution reservoir, tubing and valves inside the bath. The temperature in the bath was regulated by an immersion circulator manufactured by Fisher Scientific (Model 72). The control of the temperature was within 0.01°C. In this way, the temperature gradients within the system and crystallization problems in the tubing were minimized. The diffusion cell employed is a modification of a cell assembled by Sorrell [1981] and is shown in Fig. 2. After solutions of the desired concentrations (low and high concentrated solutions) were prepared and the system brought to the appropriate temperature, the diffusion cell was filled and the boundary sharpening procedure begun. The sharpness of the boundary could be qualitatively judged by the appearance of the fringe pattern observed during the process. When a fringe pattern of acceptable quality was obtained, flow to the diffusion cell from the solution reservoir was stopped. At this point the diffusion process is in the free diffusion mode. A timer was immediately started in order to record the difference in time between the beginning of free diffusion process and the subsequent photographic exposure of the Gouy fringe pattern. Fringe data

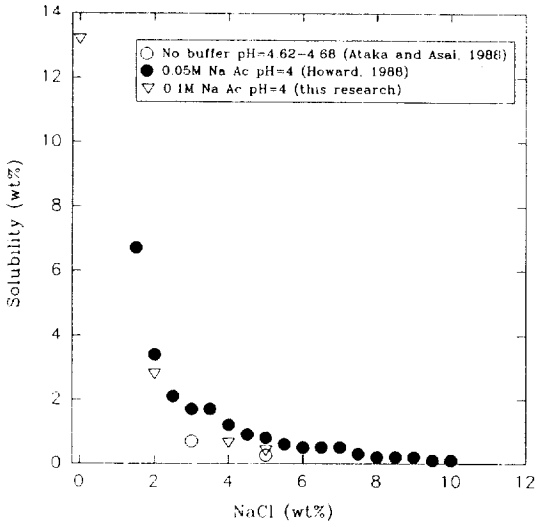
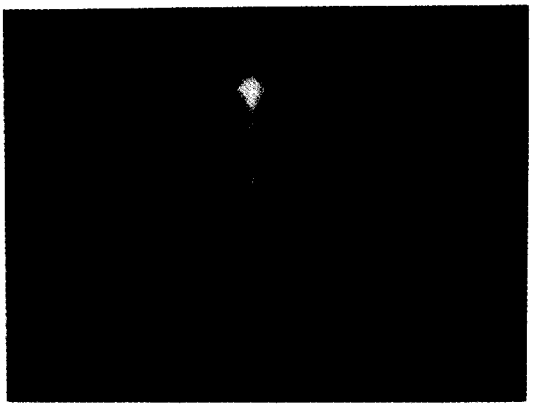
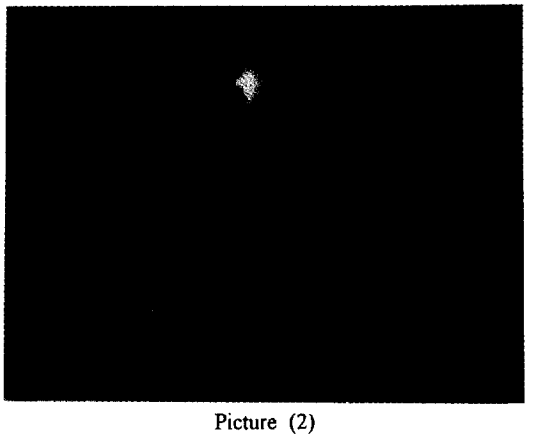


Fig. 3. Dependence of lysozyme solubility on sodium chloride concentration in various buffer solutions.



Picture (1)



Picture (2)

Fig. 4. Photographs taken from the diffusion experiment.

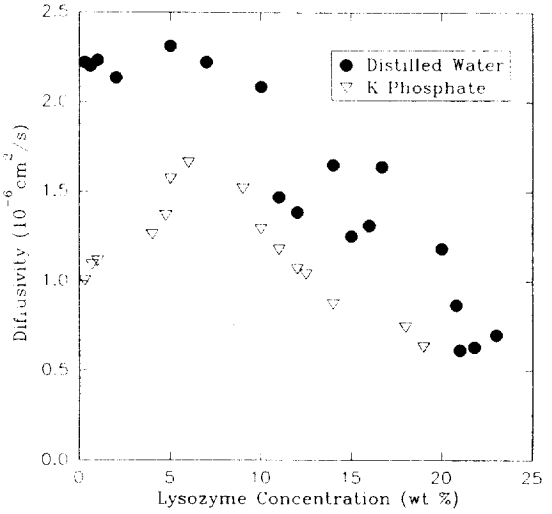


Fig. 5. Diffusion coefficient of lysozyme in distilled water (pH= 4.75) and 0.05 M potassium phosphate buffer (pH=6.8). Saturation concentrations of lysozyme in distilled water and potassium phosphate buffer are 20.8 and 14.6 wt%.

were photographically recorded employing a lensless real image camera. The distances between the undeviated slit image and the minima of the first eleven fringes were measured by the Gaertner Scientific Corporation Comparator (Model 267D). The total number of fringes present in the interference pattern was determined from the fringe photographs employing a combination of the methods described in the references [Chang, 1985; Lo, 1991]. The analysis of the photographs of the fringes yields an uncorrected diffusion coefficient for each photograph. A plot of these uncorrected diffusion coefficients versus time^{-1} extrapolated to time^{-1}=0 yields the true diffusion coefficient at the average concentration for the low and high concentrated solutions used in the diffusion cell.

RESULTS AND DISCUSSION

1. Solubility

Fig. 3 shows the solubility of lysozyme in various buffers at 25°C. For 0.1 M sodium acetate buffer the solubility of lysozyme decreased logarithmically with increasing salt concentration.

2. Diffusion Coefficient

Fig. 4 shows the fringe pattern for lysozyme taken from this diffusion experiment. The exposure time for picture (1) was longer than that for picture (2). That means fringes move upward as time passes and finally only one slit image can be obtained. From the fringe patterns the diffusion coefficients of lysozyme were calculated as a function of lysozyme concentration and plotted in Figs. 5 and 6. In Fig. 5, the diffusion coefficients of lysozyme in distilled water at pH=4.75 and 0.05 M potassium phosphate buffer at pH=6.8 and at 25°C were reported. For distilled water, the diffusion coefficient was measured at concentrations ranging from 0.3 to 23 wt%. The saturation concentration at 25°C was 20.8 wt%. The result for the distilled water shows a decline in the diffusion coefficient with increasing concentration, the rate of which increased at high lysozyme concentration. For 0.05 M potassium phosphate buffer, the diffusion coefficient of lysozyme was measured at concentrations ranging from 0.3 to 19 wt% (saturation concentration was 14.6 wt%). The diffusion coefficient first increased with increasing concentration until a maximum was observed at 6 wt% and then declined. This behavior was likely a result

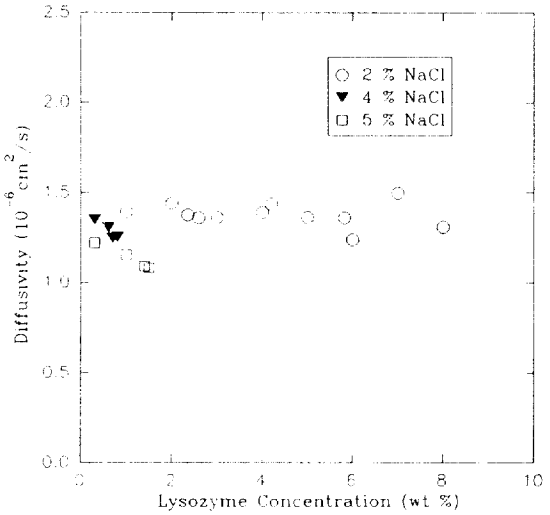


Fig. 6. Diffusion coefficient of lysozyme in 0.1 M sodium acetate buffer (pH=4.0) with 2, 4, and 5 wt% NaCl, respectively. Saturation concentrations of lysozyme in sodium acetate buffer with 2, 4, and 5 wt% NaCl are 2.79, 0.64, and 0.4 wt%.

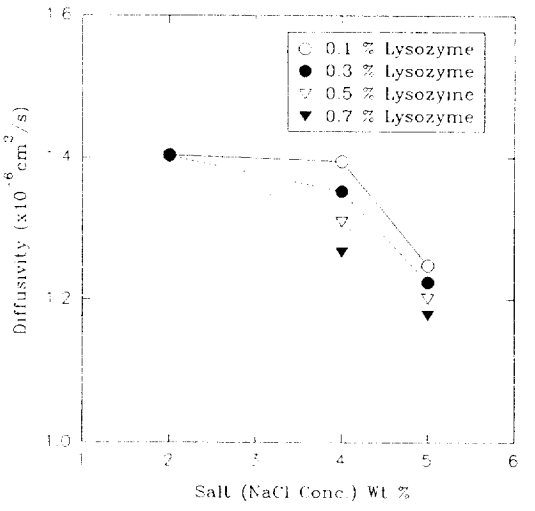


Fig. 7. Diffusion coefficient of lysozyme with salt concentration. 0.1 M sodium acetate buffer was used at 25°C and pH= 4.0. The numbers in the box denote the lysozyme concentrations.

of electrolyte interaction. A similar result was observed for ovalbumin in sodium acetate buffer (0.1 M) with small amount of NaCl (0.3 wt%) [Marlowe, 1983]. Marlowe [1983] observed behavior similar to that seen in potassium phosphate buffer. The diffusion coefficients of lysozyme in distilled water (pH= 4.75) were higher than those in 0.05 M potassium phosphate buffer (pH=6.8) over the whole range of concentration and much higher at low concentration. Lysozyme molecules have larger positive charges at lower pH and then more repulsive electrostatic interaction might be applied on the molecules. This may be the result of the small ions ionized from the buffer surrounding the macroions and reducing the effect of the electrostatic interaction.

In Fig. 6, the diffusion coefficients of lysozyme in 0.1 M sodium acetate buffer with 2, 4, and 5 wt% sodium chloride at pH=4.0 and 25°C are shown. Diffusion coefficients were measured at concentrations ranging from 1 to 8 wt% at 2 wt% NaCl (saturation concentration was 2.79 wt%), from 0.3 to 1 wt% at 4 wt% NaCl (saturation concentration was 0.64 wt%), and 0.3 to 1.5 wt% at 5 wt% NaCl (saturation concentration was 0.4 wt%). The effect of lysozyme concentration on the diffusivity in this salt-buffer system was small, but salt concentration affected diffusion coefficient significantly. An increase in salt concentration decreased the diffusion coefficient of lysozyme.

The diffusion coefficient of lysozyme in 0.1 M sodium acetate buffer with 2 wt% NaCl appeared independent of the lysozyme concentration, up to high supersaturation (the relative supersaturation=solution concentration/saturation concentration= 3). The diffusion coefficient of lysozyme at 4 and 5 wt% NaCl decreased with increasing lysozyme concentration in the supersaturated region. The diffusion behavior shown in Fig. 6 looks like that of a neutral molecule. When the salt is in a lysozyme solution, macroions can be surrounded by the ions separated from salt component and a new ionic atmosphere is formed. When macroions diffuse, the ionic atmosphere moves together. Therefore the body of ions (ionic atmosphere) can act like neutral molecules because the electrostatic interaction can be diminished by the surrounding ions. When there is no salt in the lysozyme solution, however lysozyme molecules can ionize into macroions and counterions, and act like electrolytes. In a salt-free system (as in Fig. 5), the diffusion behavior with lysozyme concentration looks like that of an electrolyte.

To examine the effect of ionic strength on the diffusion coefficient of lysozyme, the diffusion coefficients at several constant lysozyme concentrations were obtained by interpolation from each plot in Fig. 6. The results are shown in Fig. 7. The diffusion coefficient of lysozyme decreased with increasing salt concentration, i.e., ionic strength. Because it is already known that the conformational change for the globular protein, lysozyme, can be neglected with salt concentration, it is thought that the added salt affects the electrostatic interaction.

The diffusion coefficients of lysozyme reported in the literature [Azuma et al., 1989; Dubin et al., 1967; Mikol et al., 1989] are not directly comparable to the experimental values obtained in this work because of differences in the experimental conditions. Dubin et al. [1967] measured the diffusion coefficient of lysozyme in aqueous solution at 6 wt% at 25°C and pH 5.6 and obtained 1.15×10^{-6} cm^2/s. In this work the diffusion coefficient of lysozyme in aqueous solution at 6 wt% at 25°C and pH 4.75 was 2.25×10^{-6} cm^2/s. Azuma et al. [1989] measured the coefficients of lysozyme in the ranges of 1-12% w/v in aqueous solution (undersaturated region) and 0.15-2% w/v (maximum relative supersaturation=13.33) in 0.1 M sodium acetate buffer (pH=4-4.2) with 5% NaCl. Their data were scattered from 0.1×10^{-6} to 1×10^{-6} cm^2/s in aqueous solution and from 0.05×10^{-6} to 0.5×10^{-6} cm^2/s in the buffer. We obtained the values of 2.1 2.3×10^{-6} cm^2/s in aqueous solution and of 1.0 1.3×10^{-6} cm^2/s in the same buffer (pH=4.0) over the same ranges (Fig. 5). Mikol et al. [1989] obtained the diffusion coefficient of lysozyme of 1.03×10^{-6} cm^2/s in 0.04 M sodium acetate buffer (pH = 4.6) with 2 wt% NaCl at 25°C below the saturation point (about 3.1 wt%). The coefficient decreased slow-

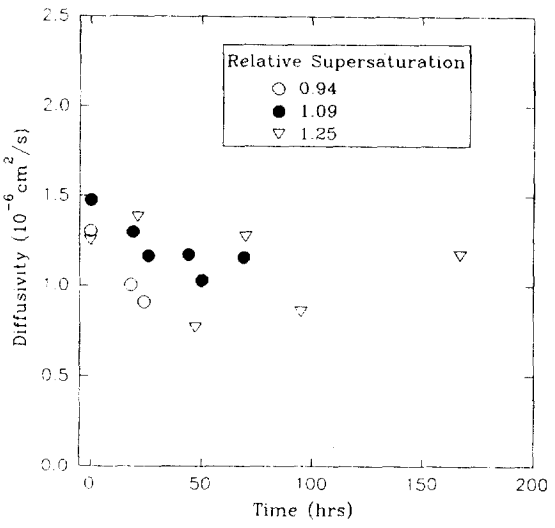


Fig. 8. Diffusion coefficient of lysozyme with solution 'Age'. 0.1 M sodium acetate buffer with 4 wt% NaCl at pH = 4 and 25°C was used.

ly in the supersaturated region. In this work the coefficient was 1.40×10^{-6} cm^2/s and remained constant with increasing lysozyme concentration into the supersaturated region.

The effect of solution 'age' on the diffusion coefficient of supersaturated lysozyme solutions in 0.1 M sodium acetate buffer with 4 wt% NaCl at 25°C and pH=4.0 is shown in Fig. 8. Diffusion coefficient appeared decreasing with increased solution 'age' before the saturation point (relative supersaturation=0.94). This may be the result of the strong molecular interactions of polyelectrolytes in solution and the formation of molecular aggregates in the undersaturated solution as reported by Pusey [1991, 1992].

CONCLUSIONS

Unlike previous work with small molecules [Chang, 1985; Chang and Myerson, 1985, 1986; Lo, 1991; Myerson and Lo, 1991; Myerson and Senol, 1984; Sorell, 1981], diffusion coefficients of lysozyme did not decline sharply with solution 'age' and with increasing concentration in supersaturated solutions. Concentration and 'age' appeared to affect the diffusion coefficient slightly, but they were small compared to the effects of salt addition.

The diffusion coefficients of lysozyme versus concentration in distilled water (pH=4.75) and 0.05 M potassium phosphate buffer (pH=6.8) showed typical electrolyte diffusion behavior. The diffusion coefficient in distilled water was always higher than that in potassium phosphate buffer over the entire range of lysozyme concentrations due to the charge effect. The difference of the diffusion coefficients for two buffer solutions became smaller at higher lysozyme concentration. The diffusion coefficient in potassium phosphate buffer reached a maximum at about 6 wt% lysozyme. Unlike small molecules, the diffusion coefficient of lysozyme began to decrease before saturation. This could be due to the formation of dimers, or trimers in the undersaturated solution due to molecular interactions as reported by Pusey [1991, 1992].

The diffusion coefficient in sodium acetate buffer with 2% NaCl did not decrease with increasing lysozyme concentration. The diffusion coefficient with 4 and 5 wt% NaCl decreased with increasing lysozyme concentration. The rate of decrease in the diffusion coefficient increased with increasing salt concentration. The diffusion coefficient of lysozyme also decreased with increasing salt concentration at constant lysozyme concentration.

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