LBNL-44257
Preprint

ERNEST ORLANDO LAWRENCE
BERKELEY NATIONAL LABORATORY

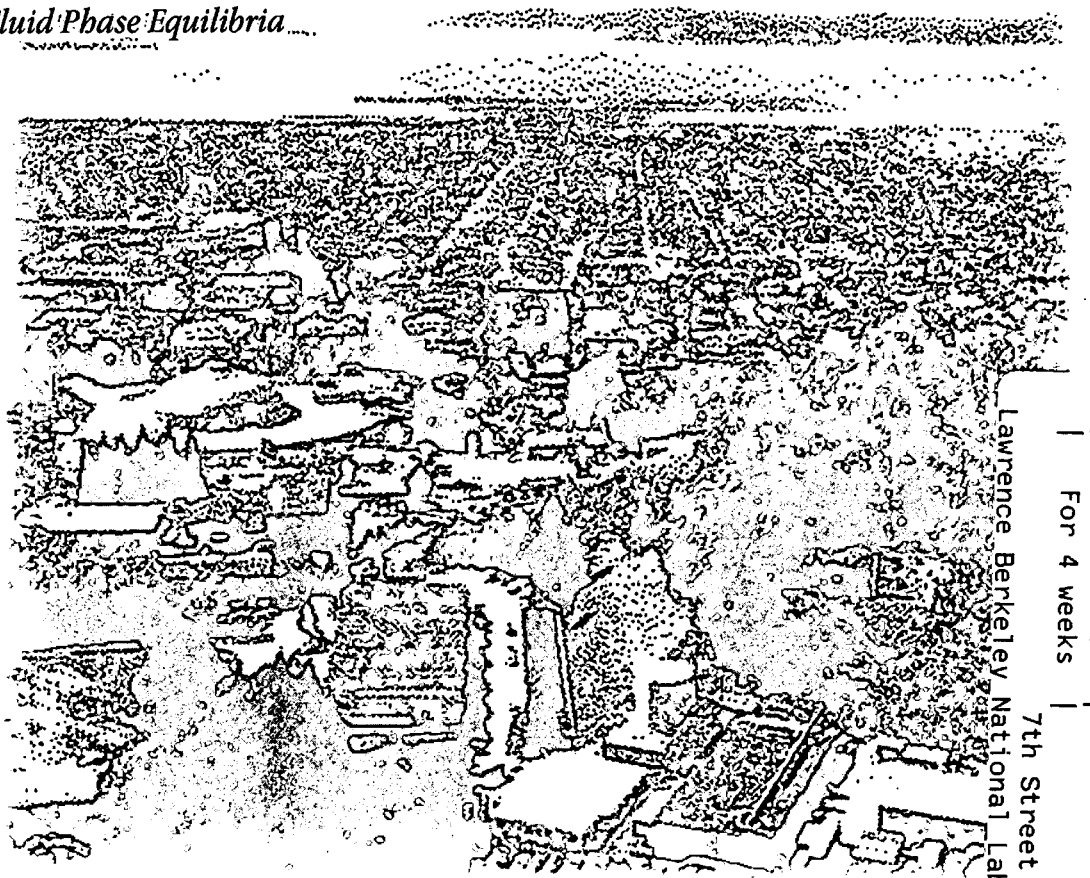
Osmotic Pressures and Second Virial Coefficients for Aqueous Saline Solutions of Lysozyme

Y.U. Moon, C.O. Anderson,
H.W. Blanch, and J.M. Prausnitz

Chemical Sciences Division

September 1999

Submitted to
Fluid Phase Equilibria



DISCLAIMER

LBNL-44257

Osmotic Pressures and Second Virial Coefficients for Aqueous Saline Solutions of Lysozyme

Y. U. Moon, C. O. Anderson, H. W. Blanch and J. M. Prausnitz
Department of Chemical Engineering
University of California
and
Chemical Sciences Division
Lawrence Berkeley National Laboratory
University of California
Berkeley, CA 94720, U.S.A.

September 1999

This work was supported by the Director, Office of Science, Office of Basic Energy Sciences, Chemical Sciences Division of the U.S. Department of Energy under Contract Number DE-AC03-76SF00098.

Osmotic Pressures and Second Virial Coefficients for Aqueous Saline Solutions of Lysozyme

Y. U. Moon, C. Anderson, H. W. Blanch and J. M. Prausnitz

Chemical Engineering Department

University of California, Berkeley

and

Chemical Sciences Division

Lawrence Berkeley National Laboratory

Berkeley, CA 94720

Abstract

Experimental data at 25°C are reported for osmotic pressures of aqueous solutions containing lysozyme and any one of the following salts: ammonium sulfate, ammonium oxalate and ammonium phosphate at ionic strength 1 or 3M. Data were obtained using a Wescor Colloid Membrane Osmometer at lysozyme concentrations from about 4 to 20 grams per liter at pH 4, 7 or 8. Osmotic second virial coefficients for lysozyme were calculated from the osmotic- pressure data. All coefficients were negative, increasing in magnitude with ionic strength. Results are insensitive to the nature of the anion, but rise slightly in magnitude as the size of the anion increases.

Introduction

In the biotechnology industry, protein precipitation is widely used to recover and purify proteins from aqueous solutions using inorganic salts or nonionic polymers as a precipitating agent (Rothstein, 1994).

When typical salts are added to water in appreciable amounts, the structure of water is altered. Enhanced association between water and salt ions causes water to be stripped from the protein, thereby increasing hydrophobic attraction between protein molecules (Becker, 1995; Coen, 1995; Vlachy, 1992, 1993). Salts with these properties are called kosmotropes; they are effective at salting-out proteins. Due to their high charge density and small size, kosmotropes increase the surface tension of water and serve as water-structure makers. Ions that break water structure, called chaotropes, tend to salt-in proteins. The surface tension of water is lowered when large ions disturb the water lattice (Rothstein, 1994). The salting-out and salting-in influence of ions was recognized long ago by Hofmeister (1888) who proposed a qualitative lyotropic series.

For common anions, the Hofmeister series is given by $SO_4^{2-} > H_2PO_4^- > CH_3COO^- > Cl^- > Br^- > I^- > NO_3^- > SCN^-$; for common cations it is $Li^+ > Na^+ > K^+ > NH_4^+$. Generally, ions high in the lyotropic series act as kosmotropes, ordering the structure of water and causing increased attraction between protein molecules. As more salt is added, increased protein-protein attraction leads to precipitation. Ions low in the series (chaotropes) often act as salting-in agents.

An osmotic-pressure system consists of two cells separated by a semi-permeable membrane. The system contains an inner aqueous saline solution (component 1) and protein (component 2), and an outer solution containing only component 1. The inner

solution is denoted as the sample solution; the outer solution is the reference solution. The membrane that separates these two solutions is impermeable to the protein but permeable to water and salt. At equilibrium, the chemical potential of any permeable species must be equal on both sides of the membrane. The presence of a non-diffusible solute in the sample solution reduces the solvent chemical potential and causes solvent from the reference chamber to the sample chamber. Solvent flow can be eliminated and equilibrium established by applying additional pressure to the sample chamber.

The additional pressure is the osmotic pressure Π as discussed in texts on physical chemistry. To express Π in terms of protein concentration, we utilize the solution theory of McMillan and Mayer (1945) who applied the theory of imperfect gases to dilute solutions of a solute in a liquid solvent. The McMillan-Mayer osmotic virial equation is

$$\frac{\Pi}{\mathbf{kT}} = \rho_2 + \mathbf{B'}_{22}\rho_2^2 + \mathbf{B'}_{222}\rho_2^3 + \cdots\cdots \qquad (1)$$

where ρ_2 is the number density of protein molecules, **k** is Boltzmann's constant and **T** is absolute temperature. $\mathbf{B'}_{222}$ is the osmotic third virial coefficient. The osmotic second virial coefficient $\mathbf{B'}_{22}$ is related to the potential of mean force $\mathbf{W}_{22}$:

$$\mathbf{B'}_{22} = -\frac{1}{2}\int_0^\infty [\mathbf{e}^{-\mathbf{W}_{22}/\mathbf{kT}} - 1]4\pi\mathbf{r}^2\mathbf{dr} \qquad (2)$$

where **r** is the center-to-center distance between two protein molecules. equation (1) can be converted into an expansion in protein mass concentration $\mathbf{c}_2$ with the relationship $\rho_2=\mathbf{c}_2\mathbf{N}_A/\mathbf{M}_2$:

$$\frac{\Pi}{\mathbf{c}_2\mathbf{RT}} = \frac{1}{\mathbf{M}_2} + \mathbf{B}_{22}\mathbf{c}_2 + \mathbf{B}_{222}\mathbf{c}_2^3 + \cdots\cdots \qquad (3)$$

where **R** is the universal gas constant, and $\mathbf{M_2}$ is the protein molecular weight. The osmotic second virial coefficient in equation (3) is given by

$$\mathbf{B_{22}} = \frac{\mathbf{B'_{22}}}{\mathbf{M_2^2}} \mathbf{N_A} \qquad (4)$$

When $\mathbf{c_2}$ is small, the last term in equation (3) can be neglected. When the osmotic pressure is measured over a range of low concentrations, the osmotic second virial coefficient $\mathbf{B_{22}}$ and the molecular weight $\mathbf{M_2}$ can be determined. When $\mathbf{B_{22}}$ is positive, the net interaction between protein molecules is repulsive; when $\mathbf{B_{22}}$ is negative, the net interaction between protein molecules is attractive.

Protein-Protein Interaction

The importance of protein-protein interactions in protein crystallization has been demonstrated by George and Wilson (1994) who have proposed that a crystallization "window" exists for the protein-protein osmotic second virial coefficient, B_{22}, which is a direct measure of the protein-protein pair potential. Protein-Protein interactions can be probed by a variety of techniques including membrane osmometry, sedimentation, and low-angle laser scattering (LALLS). All of these techniques yield a protein-protein osmotic second virial coefficient (B_{22}), that can be related to the potential of mean force.

The potential of mean force is defined such that its negative derivative with respect to distance is the force between two solute molecules at infinite dilution, averaged over all configurations of the solvent molecules (McMillan and Mayer, 1945). If the potential of mean force (W_{22}) is for a globular protein, a possible expression for W_{22} is the sum of the following spherically symmetric potentials

$$W_{22}(r) = W_{hs}(r) + W_{elec}(r) + W_{disp}(r) + W_{osmotic}(r) \qquad (5)$$

where r is the center-to-center distance, $W_{hs}(r)$ is the protein hard-sphere (excluded-volume) potential, $W_{elec}(r)$ is the electric double-layer repulsion potential, $W_{disp}(r)$ is the dispersion potential of Hamaker, $W_{osmotic}(r)$ is an attractive interaction due to the excluded-volume effect of the salt ions. The first three terms, $W_{hs}(r)$, $W_{elec}(r)$, and $W_{disp}(r)$, are described by Derjaguin-Landau-Verwey-Overbeek (DLVO) theory (Verwey and Overbeek, 1948) where proteins are modeled as rigid spheres with uniform surface charge immersed in a continuous dielectric medium containing point-charges salt ions. Details concerning equation (5) are given elsewhere (Vlancy et al, 1993) Osmotic pressures for some proteins at low concentrations of univalent salts may be predicted accurately by the DLVO model (Wu and Prausnitz, 1998; Coen et al., 1995; Vilker et al., 1981). However, at higher salt concentrations, the excluded volume of the salt ions is significant and $W_{osmotic}(r)$ must be included in the model (Vlancy et al, 1993). Size parameters are given in Table 4.

In our studies on the fundamentals of protein precipitation using salts, we were struck by the observation of George and Wilson (1994, 1996) that indicate a relationship between precipitation effectiveness and osmotic second virial coefficient: A good crystallizing agent is likely to be one that produces a moderately negative osmotic second virial coefficient of the protein at a reasonable salt concentration well below saturation. We have therefore measured the osmotic pressures of aqueous salt solutions of lysozyme at 25 °C in the protein concentration range 4 to 20 grams per liter at three pH: 4, 7 and 8. Three ammonium salts were studied: sulfates, oxalates, and phosphates at two levels of salt concentration corresponding to ionic strength 1 and 3 M.

Experimental

Materials

Hen-egg-white lysozyme was purchased from Boehringer Mannheim Corporation GmbH (Germany). Ammonium Sulfate, and Dibasic Ammonium Phosphate certified ACS were purchased from Fisher Scientific company (Fair Lawn, NJ), and Ammonium Oxalate was purchased from Sigma Company (St. Louis, MO). A Barnstead-Nanopure Water-Purification System was used to purify the water used in all experiments. Regenerated cellulose membrane disks with a nominal molecular weight cutoff of 10,000 were purchased from Millipore Corporation (Marlborough, MA). Membranes were soaked in deionized water overnight and soaked in 0.9% NaCl solution for three nights before use.

A bulk lysozyme solution with an approximate concentration of 20g/L was prepared by dissolving the protein in a salt solution with the desired ionic strength. Ionic strength I is defined by:

$$I = \frac{1}{2}\sum_i m_i \cdot z_i^2$$

where m_i is the molarity of ion i and z_i is the ion's charge. For most solutions, the pH was adjusted using ammonium hydroxide and sulfuric acid of the same ionic strength as that of the protein solution. However, for ammonium oxalate solutions, to adjust to pH 4, 20 N sulfuric acid was used. The pH meter was from Corning Incorporated (model pH 340, series No.: C4668). To remove any precipitate and bubbles, the lysozyme solution was filtered using Sterile Millex-GS, a 0.22μm-filter unit purchased from Millipore

Company (Bedford, MA). The 20g/L-lysozyme solution was diluted with the corresponding salt solution to obtain nine 5-ml samples ranging in concentration from 4 to 20g/L.

The concentration of each sample solution was measured using a Beckman DU-6 Spectrophotometer (Beckman Instruments Incorporation, Series No.: 4135285) at a wavelength of 280 nm. The measured extinction coefficient of the lysozyme solution depends on the salt. It was 2.43 L/g·cm for ammonium sulfate and ammonium phosphate solutions and 2.36 L/g·cm for ammonium oxalate solutions. The extinction coefficient is independent of pH and ionic strength for the conditions investigated here.

Membrane Osmometer

Osmotic-pressure measurements were made using a Wescor Colloid Osmometer (model 4420, Logan, UT). Figure 1 shows a schematic of the osmotic-pressure system. Calibration of this instrument was carried out using a water manometer made by Wescor company (Logan, UT).

The reference solution is contained in the lower cell while the upper cell contains the protein solution. Syringes are used to inject about 10 ml salt solution into the reference chamber and 5 ml protein solution into the sample chamber. These amounts ensure that solutions from the previous measurement are completely flushed out.

Results

Measurements with lysozyme in ammonium sulfate solution were taken over an ionic-strength range of 1.0 to 3.0 M at pH 4, 7, and 8. Measurements in ammonium phosphate solution were taken over an ionic strength range of 1.0 to 3.0 M at pH 7, and 8. In ammonium oxalate solution, measurements were taken at 1.0 M ionic strength at pH 4, 7, and 8. Table 1 shows measured osmotic pressures. In our method of data reduction, we did not consider salt as a component. We considered the protein as the solute and salt solution as the solvent. The osmotic second virial coefficient is obtained from the slope of a plot of Π/c_2RT versus c_2. The inverse of the intercept is the molecular weight at infinite dilution. An illustrative plot is shown in Figure 2 that presents typical data for osmotic pressures of lysozyme in ammonium sulfate. For pH 8.0 and I=1.0 M ammonium sulfate, the infinite-dilution molecular weight is 17000 g/mol, and the osmotic second virial coefficient is -3.07×10^{-4} ml-mol/g^2. Figure 3 presents number-average molecular weights and osmotic second virial coefficients ($\mathbf{B}_{22}$). Table 2 shows that the second virial coefficient of lysozyme becomes more negative as pH increases from 4 to 8. All osmotic second virial coefficients are negative indicating net attraction of lysozyme molecules in solution. The magnitude of the coefficient rises with increasing ionic strength.

The electric charge on lysozyme depends on pH as shown in Table 3. As pH rises, the charge declines. It is therefore reasonable that the osmotic second virial coefficient becomes more negative as the pH goes from 4 to 8. The osmotic second virial coefficient of lysozyme depends on ionic strength as shown in Figure 4.

Table 5 shows that regressed Hamaker constants for lysozyme depend slightly on salt concentration. The reduced Hamaker constant ranges from 6.4 to 8.4, in reasonable agreement with previous studies (Eberstein et al., 1994) of lysozyme. Hamaker constants are smaller in the more concentrated salt solution indicating that the osmotic contribution to the potential of mean force model is over-predicting the osmotic attraction.

For the three ammonium salts studied here, the anion has little influence on the osmotic second virial coefficient of lysozyme. The magnitude of this coefficient rises slightly with increasing anion size. The osmotic second virial coefficients observed here are in reasonable agreement with those in similar salt solutions reported previously. As suggested by the work of George and Wilson (1994), any one of the ammonium salts studied here is suitable for crystallization of lysozyme, especially at ionic strength 3 M.

Acknowledgement

For financial supports the authors are grateful to the National Science Foundation and to the Office for Basic Energy Research of the US Dept. of Energy.

Literature Cited

Becker, T., "Separation and Purification Process for Recovery of Industrial Enzymes," in Bioseparation process in foods, R. K. Singh and S. S. H. Rizvi, Eds., Marcel Dekker, Inc., New York (1995).

Coen, C. J., H. W. Blanch and J. M. Prausnitz, "Salting Out of Aqueous Proteins: Phase Equilibria and Intermolecular Protenials" AIChE. **41**, 996 (1995).

Eberstein, W., Georgalis, Y., Saenger, W., "Molecular Interactions in Crystallizing

lysozyme solutions studied by photon correlation spectroscopy" J. Cryst. Growth, **143**, 71(1994).

George, A. and Wilson W. W, "Predicting Protein Crystallization from a Dilute Solution Property" Acta Cryst. **D50**, 361 (1994).

Hofmeister, F., "Zur Lehre von der Wirkung der Salze," Arch. Pathol. Pharmakol, **24**, 247 (1888).

Kuehner, D. E., Heyer, C. et al., "Interactions of Lysozyme in Concentrated Electrolyte Solutions from Dynamic Light-Scattering Measurements" Biophysical J., **73**, 3211 (1997).

Marcus, Y., " A simple Empirical Model Describing the Thermodynamics of Hydration of Ions of Widely Varying Charges, Sizes, and Shapes" Biophysical Chemistry, **51**, 111(1994).

McMillan Jr., W.G. and J. E. Mayer, "The Statistical Thermodynamics of Multicomponent Systems" J. Chem. Phys. **13**, 276 (1945).

Rothstein, F., "Differential Precipitation of Proteins" Protein Purification Process Engineering, R.G.Harrison, ed., Marcel Dekker, New York p. 115 (1994).

Verwey, E. J. W., Overbeek, J. T. K., "Theory of Stability of Lyophobic Colloids" Elsevier, Amsterdam (1948).

Vlachy, V., H.W. Blanch, and J. M. Prausnitz, "Liquid-Liquid Phase Separations in Aqueous Solutions of Globular Proteins" AIChE J. **39**, 215 (1993).

Wilson, L. J., Adcock-Downey, and M.L. Pusey, "Monomer Concentrations and Dimerization Constants in Crystallizing Lysozyme Solution by Dialysis Kinetics."Biophys. J. **71**, 2123 (1996).

Wu, J. Z. and J. M. Prausnitz, "Osmotic Pressures of Aqueous Bovine Serum Albumin Solutions at High Ionic Strength" Fluid Phase Equilibria, **155**, 139 (1999).

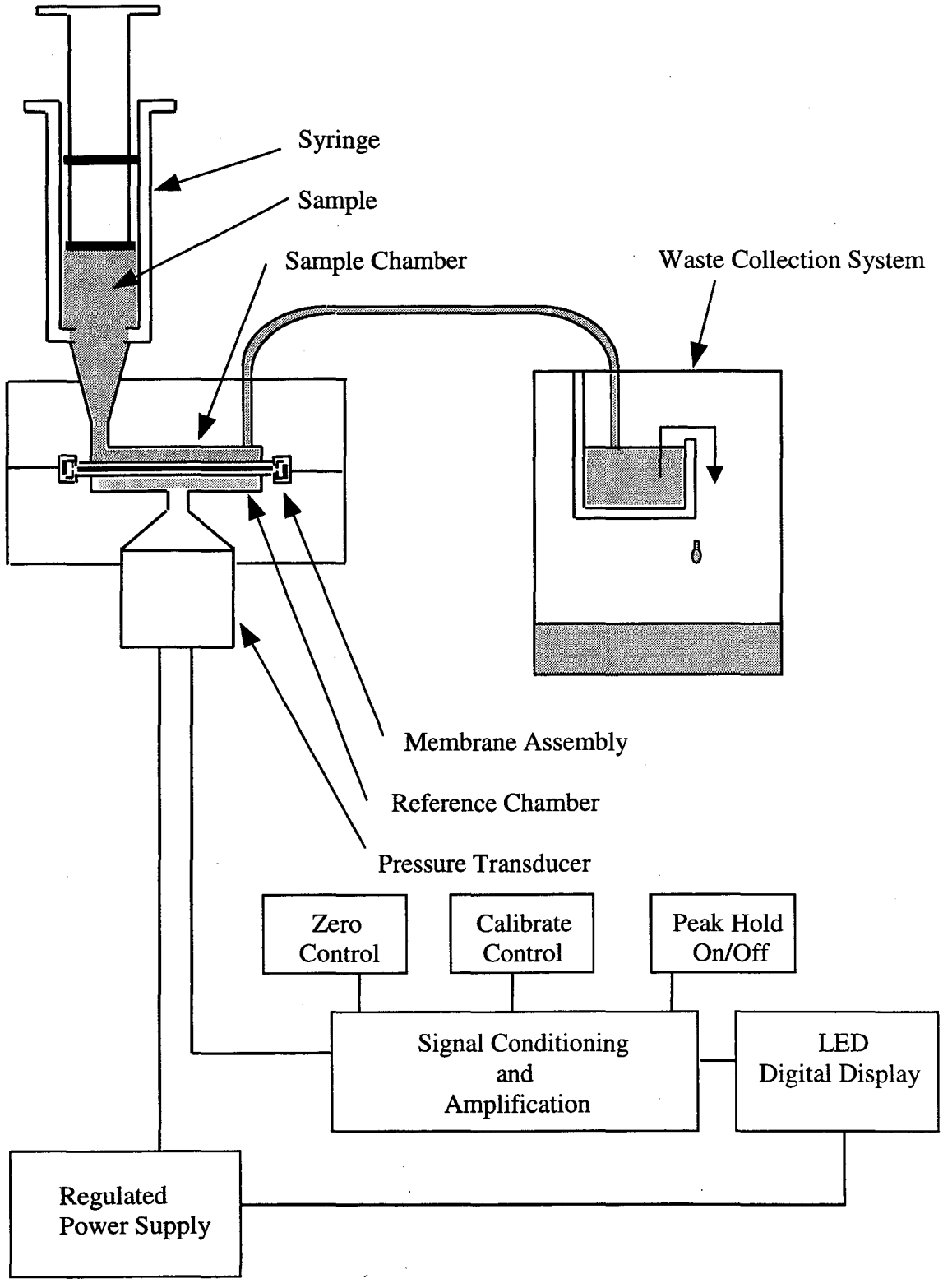


Fig. 1. Schematic Diagram of the Wescor 4420 Colloid Osmometer

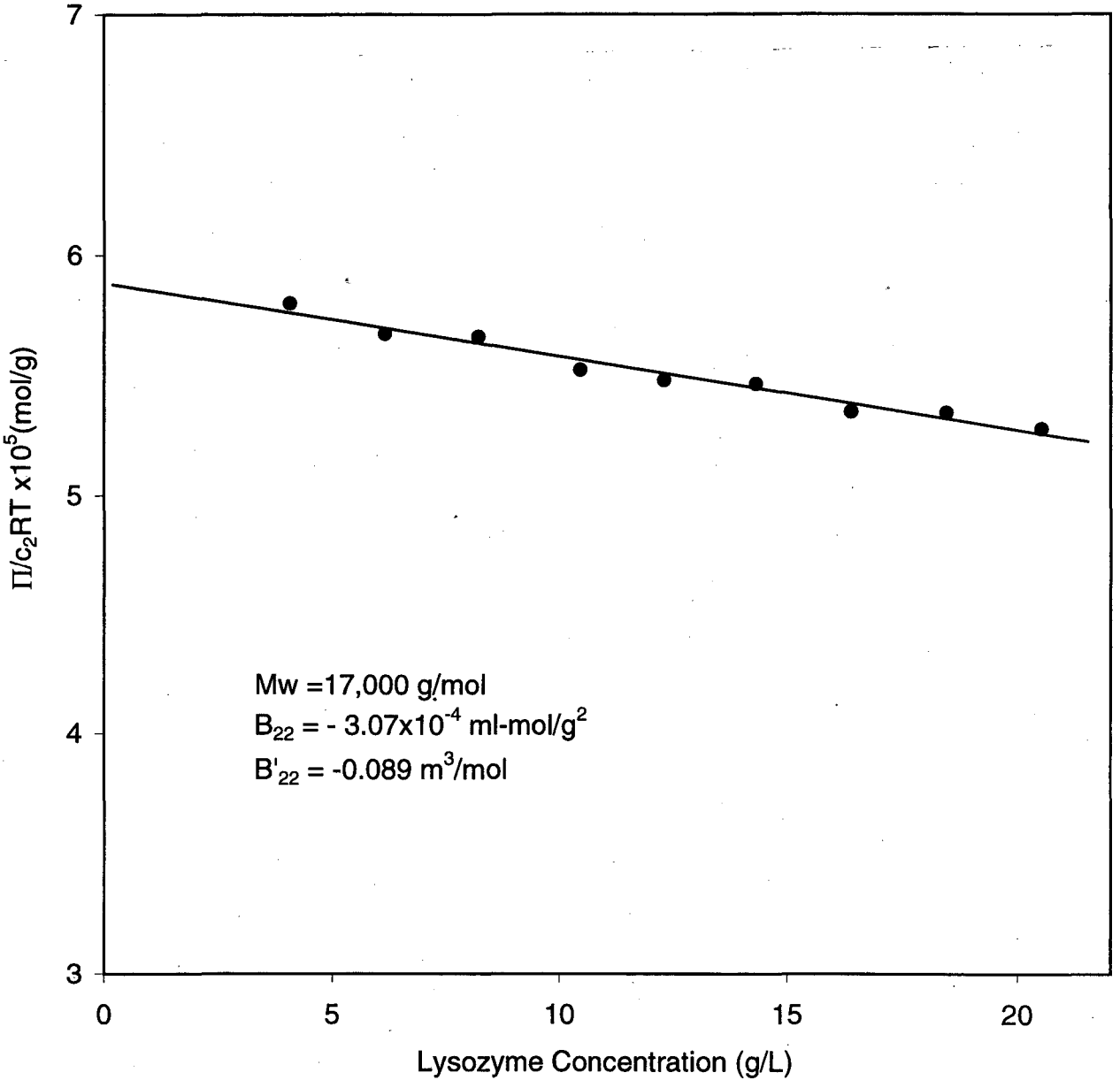


Fig. 2. Osmotic-Pressure Data for Lysozyme at pH 8.0 and 25 °C in 1.0M Ionic Strength $(NH_4)_2SO_4$ Solution.

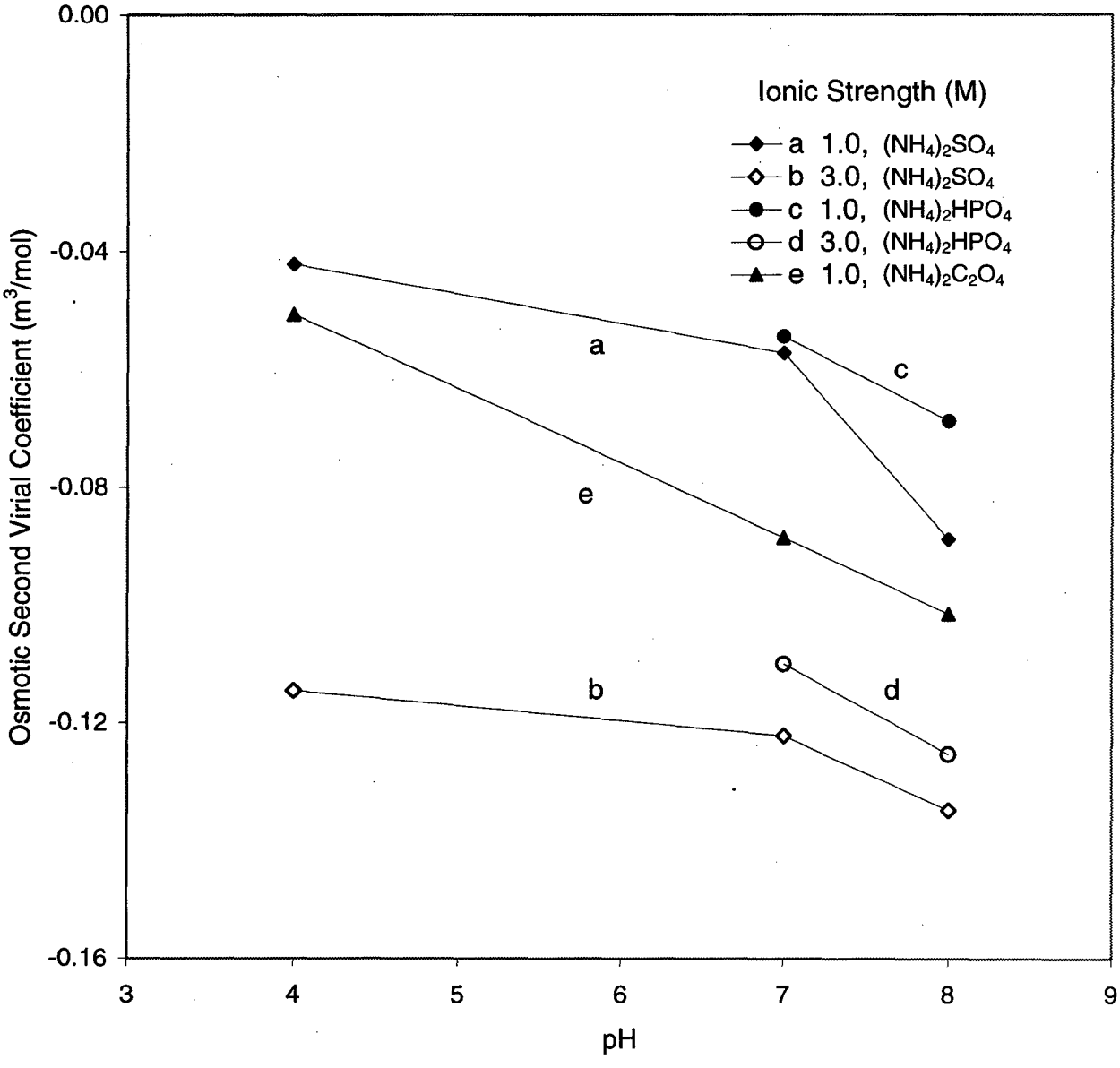


Fig. 3. Effect of pH on the Osmotic Second Virial Coefficient of Lysozyme.

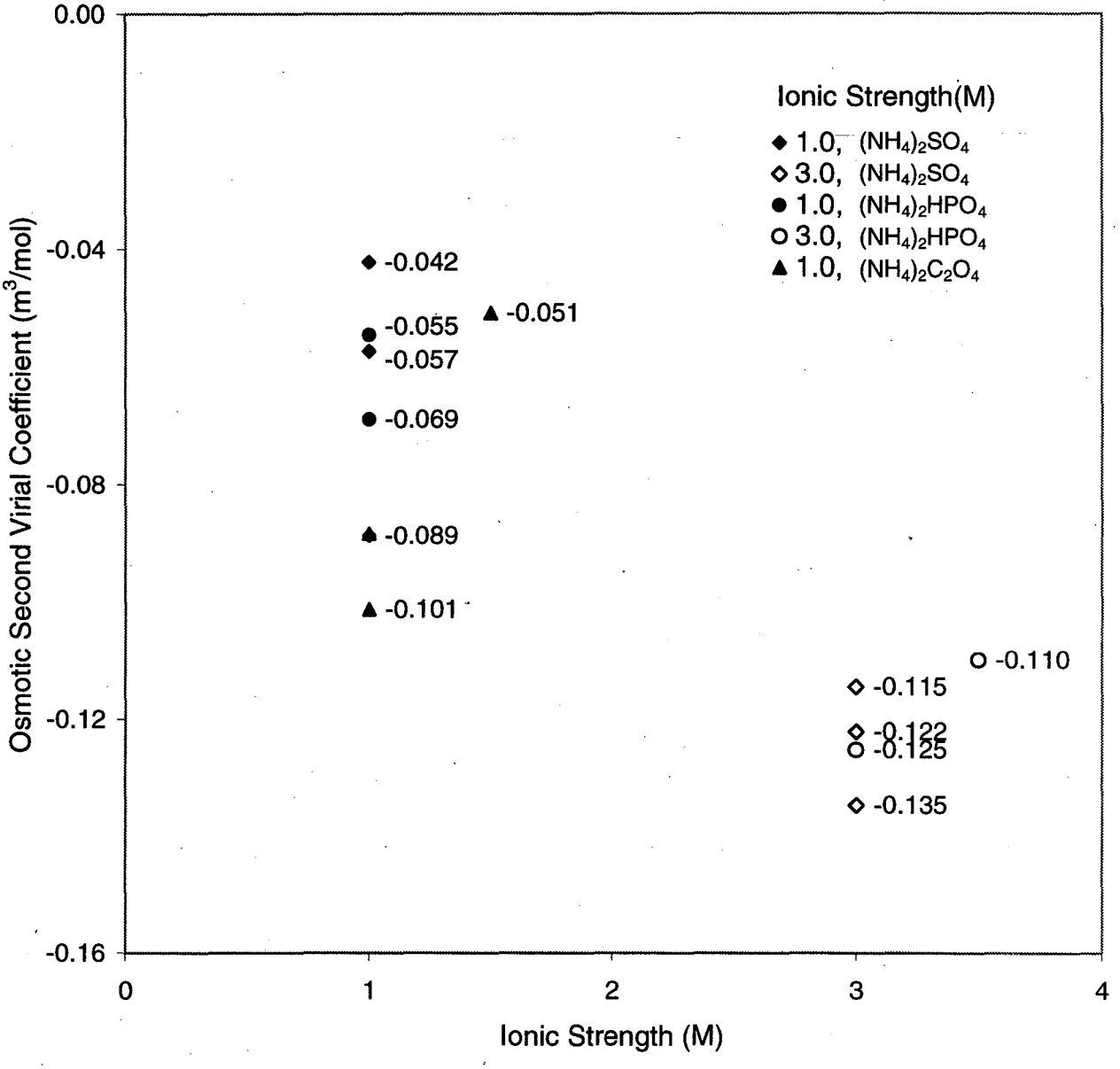


Fig. 4. Effect of Ionic Strength on the Osmotic Second Virial Coefficient of Lysozyme

Table 1. Osmotic-Pressure Data for Aqueous Salt Solutions of Lysozyme at 25°C.

Salt	$(NH_4)_2SO_4$				$(NH_4)_2C_2O_4$		$(NH_4)_2HPO_4$			
Ionic Strength (M)	1.0		3.0		1.0		1.0		3.0	
pH	c_2 (g/L)	Π (mmHg)	c_2 (g/L)	Π (mmHg)	c_2 (g/L)	Π (mmHg)	c_2 (g/L)	Π (mmHg)	c_2 (g/L)	Π (mmHg)
	4.12	4.50	4.39	4.67	4.01	4.40	-	-	-	-
	6.31	6.80	6.71	7.10	6.10	6.50	-	-	-	-
	8.31	8.90	8.89	9.15	8.12	8.70	-	-	-	-
4	10.36	11.20	11.11	11.30	10.13	10.80	-	-	-	-
	12.47	13.20	13.30	13.20	12.18	12.90	-	-	-	-
	14.46	15.30	15.54	15.40	14.24	15.10	-	-	-	-
	16.49	17.40	17.70	17.05	16.26	16.90	-	-	-	-
	18.56	19.60	19.82	18.90	18.30	19.10	-	-	-	-
	20.71	21.50	22.00	20.65	20.33	20.95	-	-	-	-
	4.06	4.45	4.30	4.63	3.94	4.25	4.07	4.43	4.09	4.40
	6.19	6.75	6.53	6.90	5.98	6.40	6.18	6.67	6.22	6.60
	8.19	8.85	8.68	8.90	7.96	8.40	8.31	8.83	8.26	8.50
7	10.25	11.00	10.85	10.90	9.91	10.30	10.37	11.00	10.27	10.50
	12.30	13.10	12.97	12.75	11.90	12.15	12.37	13.10	12.34	12.50
	14.35	15.20	15.10	14.70	13.89	14.10	14.40	15.13	14.34	14.25
	16.42	17.25	17.20	16.55	15.91	16.00	16.40	17.03	16.44	16.20
	18.42	19.23	19.32	18.30	17.96	17.90	18.41	18.98	18.41	17.70
	20.39	21.10	21.33	20.17	19.90	19.80	20.34	21.00	20.41	19.65
	4.06	4.40	4.32	4.60	3.88	4.20	4.12	4.50	4.09	4.40
	6.13	6.50	6.54	6.80	5.90	6.20	6.35	6.75	6.24	6.50
	8.18	8.65	8.88	9.00	7.85	8.30	8.36	8.85	8.28	8.50
8	10.42	10.75	10.99	11.10	9.82	10.10	10.45	11.00	10.38	10.60
	12.26	12.55	13.12	13.05	11.82	12.05	12.46	12.95	12.46	12.40
	14.26	14.55	15.28	14.70	13.83	13.80	14.55	15.00	14.58	14.10
	16.31	16.30	17.45	16.30	15.75	15.65	16.61	17.10	16.67	16.30
	18.38	18.35	19.54	18.20	17.68	17.50	18.69	19.05	18.72	17.60
	20.44	20.15	21.56	19.75	19.68	19.20	20.74	20.90	20.80	19.50

Table 2. Osmotic Second Virial Coefficients for Lysozyme at 25°C*.

Salt	Ionic Strength (M)	pH	Osmotic Second Virial Coefficient (10^{-4} ml-mol/g^2)	(m^3/mol)
$(NH_4)_2SO_4$	1	4	-1.46	-0.042
	1	7	-1.98	-0.057
	1	8	-3.07	-0.089
	3	4	-3.96	-0.115
	3	7	-4.23	-0.122
	3	8	-4.46	-0.135
$(NH_4)_2HPO_4$	1	7	-1.88	-0.055
	1	8	-2.38	-0.069
	3.5**	7	-3.81	-0.110
	3	8	-4.34	-0.125
$(NH_4)_2C_2O_4$	1.5**	4	-1.76	-0.051
	1	7	-3.06	-0.089
	1	8	-3.51	-0.101

* Average molecular weight = 17000g/mol.

** Ionic Strength changed slightly due to pH adjustment.

Table 3. Effect of pH on Net Electric Charge of Lysozyme in 1 molar Potassium Chloride Solution Obtained from Acid-Base Titration*

Ionic Strength(M)	pH	Electric Net Charge
	4	+14
1.0	7	+8
	8	+7.5

* Fergg, F., Unpublished report, University of California at Berkeley(1994)

Table 4. Lysozyme* and Ion Size** Parameters.

	Diameter, (Å)
Lysozyme	34.4
NH_4^+	2.13
SO_4^{2-}	2.78
HPO_4^{2-}	2.78
$C_2O_4^{2-}$	2.63

* Kuehner, D. E. et al., Biophysical J. (1997).

** Marcus, Y., Biophysical Chemistry (1994).

Table 5. Calculated Hamaker Constants for Lysozyme Depend Slightly on Salt Solution.

Salt	Ionic Strength (M)	pH	Reduced Hamaker Constant (H/kT)
$(NH_4)_2SO_4$	1	4	7.8
	1	7	7.3
	1	8	8.0
	3	4	6.4
	3	7	6.4
	3	8	6.5
$(NH_4)_2HPO_4$	1	7	7.2
	1	8	7.6
	3.5	7	5.6
	3	8	6.4
$(NH_4)_2C_2O_4$	1.5	4	8.4
	1	7	8.1
	1	8	8.3

Ernest Orlando Lawrence Berkeley National Laboratory
One Cyclotron Road | Berkeley, California 94720

Prepared for the U.S. Department of Energy under Contract No. DE-AC03-76SF00098