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Protein-Protein Interactions in Aqueous Ammonium Sulfate Solutions. Lysozyme and Bovine Serum Albumin (BSA)

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Abstract

Osmotic pressures have been measured to determine lysozyme-lysozyme, BSA-BSA, and lysosyme-BSA interactions for protein concentrations to 100 g/L in an aqueous solution of ammonium sulfate at ambient temperature, as a function of ionic strength and pH. Osmotic second virial coefficients for lysozyme, for BSA, and for a mixture of BSA and lysozyme were calculated from the osmotic-pressure data for protein concentrations to 40 g/L. The osmotic second virial coefficient of lysozyme is slightly negative and becomes more negative with rising ionic strength and pH. The osmotic second virial coefficient for BSA is slightly positive, increasing with ionic strength and pH. The osmotic second virial cross coefficient of the mixture lies between the coefficients for lysozyme and BSA indicating that the attractive forces for a lysozyme-BSA pair are intermediate between those for the lysozyme-lysozyme and BSA-BSA pairs. For protein concentrations less than 100 g/L, experimental osmotic-pressure data are compared with results calculated from three models: a truncated virial equation of state, the random-phase approximation with the Carnahan-Starling equation of state, and an adhesive hard-sphere (AHS) model. The AHS model provides the best fit for the osmotic-pressure data.

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Introduction

Salt-induced protein precipitation is commonly used as an initial step to purify aqueous proteins because of its selectivity and low cost (Rothstein, 1994; Becker, 1995). However, protein solubility is not well understood, and selecting optimum conditions to precipitate a target protein is difficult because solubility is governed by many factors including pH, surface hydrophobicity, surface charge distribution, size, salt type, and salt concentration (see, for example, Chiew et al., 1995). Understanding how these factors affect the solubility of a particular protein is required for developing a thermodynamic framework that can predict protein solubility in a complex aqueous mixture containing salt and biomacromolecules. The essential requirement is to determine the protein-protein interactions that govern protein solubility. In this work, we report osmotic pressures for aqueous lysozyme, for aqueous bovine serium albumin (BSA) and for an aqueous mixture of lysozyme and BSA. The aqueous solutions also contain ammonium sulfate.

Protein-Protein Interactions

The importance of protein-protein interactions in protein crystallization has been demonstrated by George and Wilson (1994) who proposed that a crystallization "window" exists for the protein-protein osmotic second virial coefficient, $\mathbf{B_{22}}$, that provides a direct measure of the protein-protein pair potential. As a necessary but not sufficient condition for protein crystallization, $\mathbf{B_{22}}$ should be in the region -2×10^{-4} and -8×10^{-4} mol-ml/g^2. For $\mathbf{B_{22}}$ more positive than -2×10^{-4} mol-ml/g^2, the protein-protein attraction is usually not sufficiently strong to form stable protein crystals. For solutions

where $\mathbf{B_{22}}$ is more negative than -8 x 10^{-4} mol-ml/g^2, amorphous precipitation is likely to occur because protein-protein attractions are so strong that the protein molecules do not have adequate time to orient themselves to form crystals before forming an amorphous agglomerate.

$\mathbf{B_{22}}$ is related to the potential of mean force, $\mathbf{W_{22}}$, 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). For globular proteins, $\mathbf{W_{22}}$ can be expressed by the sum of the following potentials (Coen et al, 1995; Curtis et al, 1998):

$$\mathbf{W_{22}}(r) = \mathbf{W_{hs}}(r) + \mathbf{W_{elec}}(r) + \mathbf{W_{disp}}(r) + \mathbf{W_{osmotic}}(r) \qquad (1)$$

where r is the center-to-center distance; $\mathbf{W_{hs}}(r)$ is the protein hard-sphere (excluded-volume) potential; $\mathbf{W_{elec}}(r)$ is the electric double-layer repulsion potential; $\mathbf{W_{disp}}(r)$ is the dispersion potential of Hamaker; and $\mathbf{W_{osmotic}}(r)$ is an osmotic-depletion interaction (Asakura and Oosawa, 1958) due to the excluded-volume effect of the salt ions. The first three terms, $\mathbf{W_{hs}}(r)$, $\mathbf{W_{elec}}(r)$, and $\mathbf{W_{disp}}(r)$, are described by Derjaguin-Landau-Verwey-Overbeek (DLVO) theory (Verwey and Overbeek, 1948) where proteins are modeled as hard spheres with uniform surface charge immersed in a continuous dielectric medium containing point charges representing salt ions. Osmotic pressures for proteins at low salt concentrations may be predicted by the DLVO model (Coen et al., 1995; Vilker et al., 1981). However, at higher salt concentrations, the excluded volume of the salt ions may be important. A possible potential for this interaction is the osmotic-attraction potential of Asakura and Oosawa (1958), $W_{osmotic}(r)$.

$W_{osmotic}(r)$ is an example of a solvation force. Solvation forces follow from indirect interactions that result from averaging over the positions of the solvent molecules. If the solvent structure adjacent to the protein surface is perturbed, than the free energy associated with the overlap of the perturbed layers is related to the solvation potential of mean force (Israelachvili, 1992). Other significant solvation forces occur between the hydrophilic surface groups of the protein molecules. These forces are called hydration forces and are believed to be repulsive because a positive free energy is required to remove tightly bound water. These interactions are poorly understood because they are related to the structured water adjacent to the protein surface; that structure is not well known due to the extensive hydrogen-bonding network of water. In concentrated electrolyte solutions, the water structure is different from that of pure water, and we expect that the hydration forces between proteins are significantly altered. In addition to hydration forces, attractive hydrophobic interactions may occur between the apolar surface groups of proteins.

The Truncated Virial Equation of State

Protein-protein interactions in solution can be measured by a variety of techniques including membrane osmometry (Vilker et al., 1981), sedimentation, and static laser-light scattering. All of these techniques yield a protein-protein osmotic second virial coefficient ($\mathbf{B'_{22}}$), that can be related to the protein-protein pair potential of mean force.

From McMillan-Mayer (1945) solution theory, the osmotic pressure of a protein solution, Π, can be related to protein number density ρ_2 by

$$\frac{\Pi}{kT} = \rho_2 + \mathbf{B'_{22}}\,\rho_2^2 + \text{ higher order terms} \qquad (2)$$

The osmotic second virial coefficient, B'_{22}, is related to the pair potential of mean force W_{22}:

$$B'_{22} = -\frac{1}{2}\int_0^\infty [e^{-W_{22}/kT} - 1]4\pi r^2 dr \qquad (3)$$

where **k** is Boltzmann's constant, **T** is the absolute temperature and **r** is the center-to-center distance between two protein molecules. Equation (2) can be converted into an expansion in protein mass concentration, $\mathbf{c_2}$ (g/L), with the relation, $\rho_2 = \mathbf{c_2 N_A / M_n}$ giving

$$\frac{\Pi}{\mathbf{c_2 RT}} = \frac{1}{\mathbf{M_n}} + \mathbf{B_{22} c_2} + \text{ higher order terms} \qquad (4)$$

where **R** is the universal gas constant, $\mathbf{M_n}$ is the protein molecular weight, and $\mathbf{N_A}$ is Avogadro's number. As $\mathbf{c_2}$ approaches zero, equation (4) reduced to the van't Hoff equation. Second virial coefficients $\mathbf{B_{22}}$ and $\mathbf{B'_{22}}$ are related by

$$\mathbf{B_{22}} = \frac{\mathbf{B'_{22}}}{\mathbf{M_n^2}} \mathbf{N_A} \qquad (5)$$

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.

In this work, osmotic-pressure measurements were made for single-protein solutions to determine the osmotic second virial coefficient for lysozyme-lysozyme interactions, $\mathbf{B_{22}}$, and for BSA-BSA interactions, $\mathbf{B_{33}}$. From osmotic-pressure measurements for a mixture of lysozyme and BSA, the osmotic second virial cross coefficient, $\mathbf{B_{23}}$, was calculated using the truncated virial equation of state for the mixture (Kurata, 1982):

$$\frac{\Pi}{\mathbf{RT}} = \frac{\mathbf{c_2}}{\mathbf{M_{n2}}} + \frac{\mathbf{c_3}}{\mathbf{M_{n3}}} + \mathbf{B_{22} c_2^2} + \mathbf{B_{33} c_3^2} + \mathbf{2 B_{23} c_2 c_3} \qquad (6)$$

where $\mathbf{c_2}$ and $\mathbf{c_3}$ are mass concentrations (g/L) of lysozyme and BSA, respectively.

Mass concentrations are converted to solvent-free weight fractions for calculating B_{23}, where the weight fractions are given by $w_2 = c_2 / c_T$ and $w_3 = c_3 / c_T$. Here c_T is the total protein concentration (g/L) of the mixture. With these substitutions, equation (6) can be rewritten

$$\frac{\Pi}{c_T RT} = \frac{1}{\overline{M}_n} + (B_{22} w_2^2 + B_{33} w_3^2 + 2B_{23} w_2 w_3) c_T \qquad (7)$$

The osmotic second virial cross coefficient B_{23} is obtained from the slope of a plot of $\Pi / c_T RT$ versus c_T at constant w_2 and w_3 using the experimental values of B_{22}, B_{33}, M_{n2} and M_{n3}. The intercept of this plot gives the number average molecular weight, $\overline{M}_n$.

Random Phase Approximation (RPA) Model

The truncated virial equation of state is exact in the limit of dilute solutions. However, for protein concentrations greater than about 20 – 40 g/L, we expect significant higher-order terms in the virial equation of state. Instead of using higher-order terms in the expansion, we may use a simpler equation of state called the Random Phase Approximation. It has been used previously to model phase transitions and structure factors of colloidal solutions, and to describe phase separation of proteins due to addition of polymers or salt (Grimson, 1983; Vlachy et al., 1993). In the RPA model, an assembly of hard spheres is used as the reference system, while the remaining spherically-symmetric interactions provide perturbations. The RPA equation of state is written as a sum of reference and perturbation terms; compressibility factor Z is given by

$$Z = \frac{\Pi}{\rho kT} = \left(\frac{\Pi}{\rho kT}\right)_{ref} + \frac{\rho U}{2kT} \qquad (8)$$

where ρ is the total protein number density and **U** is the perturbation energy per unit density.

For a single-protein system containing protein **i**

$$U_i = 4\pi \int W_{ii}^{(p)}(r) r^2 dr \qquad (9)$$

where $W_{ii}^{(p)}(r)$ is the sum of the perturbation terms of the potential of mean force between two protein molecules **i**. For a system containing two or more proteins, the perturbation coefficient (U_m) is given by

$$U_m = 4\pi \sum_i \sum_j x_i x_j \int W_{ij}^{(p)}(r) r^2 dr \qquad (10)$$

where $x_i = \rho_i / \rho$. Here, $\rho = \sum_i \rho_i$. $W_{23}^{(p)}$ is the sum of perturbation terms of the potential of mean force for the cross interaction **2-3**.

The equation of state for the reference system of hard spheres is given by the Carnahan-Starling (Carnahan et al., 1969) equation. For the single-protein system, the reference term is:

$$\left(\frac{\Pi}{\rho kT}\right)_{ref} = \frac{1+\eta+\eta^2-\eta^3}{(1-\eta)^3} \qquad (11)$$

where η is the packing fraction given by

$$\eta = (\pi\rho/6)\sigma^3 \qquad (12)$$

where σ is the hard-sphere diameter of a protein molecule. For the multi-protein system, the reference system is given by the Boublik-Mansoori-Carnahan-Starling equation (Mansoori et al, 1971):

$$\left(\frac{P}{\rho kT}\right)_{ref} = \left[\frac{1}{1-Q_3} + \frac{3Q_1Q_2}{Q_0(1-Q_3)^2} + \frac{Q_2^3(3-Q_3)}{Q_0(1-Q_3)^3}\right] \qquad (13)$$

where Q_k is given by

$$Q_k = (\pi/6)\sum_i \rho_i \sigma_i^k \qquad (14)$$

where the sum is over all protein species **i.** In Eq (14), k=0, 1, 2, 3.

Adhesive Hard-Sphere (AHS) Model

Recent studies have shown that a hard-sphere repulsion and a short-range attraction in the form of Baxter's adhesive hard-sphere potential (1968) can describe protein-protein interactions. The advantage of this potential is that only one parameter is needed to describe the characteristic decay length and the magnitude of the attractive interaction. The adhesive potential is a uniform square well taken in the limit of zero well width and infinite depth while holding the area constant. The adhesive hard-sphere (AHS) potential $\Gamma(\mathbf{r})$ (Baxter, 1968) is given by

$$\frac{1}{kT}\Gamma_{22}(r) = \begin{cases} +\infty & 0 < r < \sigma_{22} \\ \ln \dfrac{12\tau_{22}(a_{22}-\sigma_{22})}{a_{22}} & \sigma_{22} < r < a_{22} \\ 0 & a_{22} < r \end{cases} \qquad (15)$$

where **r** is the center-to-center distance between the particles, σ is the protein diameter, **a** is the range of protein interaction, **k** is Boltzmann's constant and **T** is absolute temperature. The potential is taken in the limit as $a \to \sigma$ with τ and σ held fixed. τ^{-1} is a measure of the attractive interaction and sets the ratio of the interaction strength to the thermal energy. Substitution of equation (15) into equation (3) for the second virial coefficient B_{22} yields

$$B'_{22} = \frac{2}{3}\pi\sigma_{22}^3(1 - 2/3\tau_{22}) \qquad (16)$$

For an assembly of spheres interacting with the adhesive hard-sphere potential, an expression for the correlation function has been obtained in the Percus-Yevick approximation making it possible analytically to calculate the thermodynamic properties of this system. For example, the adhesive hard-sphere potential model was used by Piazza (1999) to fit accurately the osmotic compressibility of lysozyme solutions over a large concentration range (to 330 g/L) for a set of temperatures. Furthermore, Rosenbaum et al., (1996) mapped the experimental solubility curve for lysozyme on the theoretical phase diagram for the adhesive hard-sphere fluid by relating the adhesive parameter to the measured osmotic second virial coefficient $\mathbf{B_{22}}$ using equation (16).

For the single-protein system, the compressibility equation of state gives the osmotic pressure (Barboy, 1994):

$$\frac{\Pi}{\mathbf{kT}\rho} = \frac{1+\eta+\eta^2}{(1-\eta)^3} - \eta\lambda\frac{18(2+\eta)-\eta\lambda^2}{36(1-\eta)^3} \qquad (17)$$

where η is packing fraction and paramater λ is a dimensionless number that is a function of packing fraction, diameter σ and energy parameter τ.

For the single-protein system λ is given by

$$\lambda = 6-\tau+\tau\eta^{-1} - \left[(6-\tau+\tau\eta^{-1})^2 - 6(1+2\eta^{-1})\right]^{1/2} \qquad (18)$$

The first term on the right-hand side of equation (17) is the hard-sphere compressibility factor and the second is due to adhesion; it disappears when τ tends to infinity $(\lambda \to 0)$.

Barboy and Tenne (1979) solved the Percus-Yevick approximation for a binary mixture of different-sized, sticky hard spheres where the potentials are given by

$$\frac{1}{kT}\Gamma_{ij}(r) = \begin{cases} +\infty & 0 < r < \sigma_{ij} \\ \ln \dfrac{12\tau_{ij}(a_{ij} - \sigma_{ij})}{a_{ij}} & \sigma_{ij} < r < a_{ij} \\ 0 & a_{ij} < r \end{cases} \qquad (19)$$

Here, σ_{ij} is the mean protein diameter, τ_{ij} is the adhesiveness parameter for the **i-j** interaction, and the potential is taken in the limit $a_{ij} \to \sigma_{ij}$. The equation of state for the sticky hard-sphere mixture was obtained using any one of four routes: (1) the compressibility equation, (2) the virial equation, (3) the energy equation, and (4) the zero-separation theorem. The compressibility equation, the virial equation, and the energy equation all lead to the same second and third virial coefficient due to adhesion. For convenience, here we use the result obtained using the virial equation of state.

Experimental

Materials

Hen-egg-white lysozyme was obtained from Boehringer Mannheim GmbH (Germany). Bovine Serum Albumin (BSA, Fract V, Cold Alcohol Precipitated, Biotech Grade) and Ammonium Sulfate certified ACS were obtained from Fisher Scientific Company (Fair Lawn, NJ). A Barnstead-Nanopure water-purification system was used to purify water in all experiments. Regenerated Cellulose membrane disks with a nominal molecular weight cutoff of 10,000 dalton were obtained 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 and a bulk BSA solution with an approximate concentration of about 100 and 30 g/L, respectively, were

prepared by dissolving the protein in 1.0 and 3.0 M ionic-strength ammonium sulfate solutions. pH was adjusted using ammonium hydroxide and sulfuric acid of the same ionic strength as that of the protein solution. The pH meter is from Corning Incorporated (Model pH 340, Series No.: C4668).

To remove precipitates and bubbles, the lysozyme and BSA solution were filtered using Sterile Millex-GS, 0.22 μm-filter units from Millipore Company (Bedford, MA). The lysozyme and BSA solutions were diluted with the corresponding salt solution to obtain seven 5-ml samples that range from 0 to 100 g/L for 1.0 M ionic strength and five 5-ml samples that range from 0 to 30 g/L for 3.0 M ionic strength. The concentrations of lysozyme and BSA solutions were measured using a Beckman DU-6 Spectrophotometer (Beckman Instruments Incorporation, Series No.: 4135285) at wavelengths 280 nm for lysozyme and 278 nm for BSA. The measured extinction coefficients of lysozyme and BSA were 2.43 L/g·cm and 0.66 L/g·cm for ammonium-sulfate solution. For the conditions used here, the extinction coefficient does not depend on pH or ionic strength. The protein-mixture solution was made by making our stock solution of lysozyme and another of BSA at concentrations from 0 to 100 g/L for 1.0 M ionic strength and from 0 to 30 g/L for 3.0 M ionic strength. To form a mixture, the two stock solutions (at the same ionic strength) were mixed with a 1:1 volume ratio.

Membrane Osmometer

Osmotic-pressure measurements were made using a Wescor Colloid Osmometer (model 4420, Logan, UT). Calibration of this instrument was carried out using a water manometer made by Wescor Company (Logan, UT). The reference solution is placed into

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

Results

We have measured osmotic pressures of lysozyme, of BSA and of their mixture at 25 °C in the protein concentration range from 0 to 100 and from 0 to 30 g/L for 1.0 and 3.0 M ionic-strength ammonium sulfate solutions at pH 4, 6, 7 and 8. Table 1 shows detailed osmotic-pressure data for lysozyme, for BSA and for the mixture.

Figure 1 shows typical data for osmotic pressures of lysozyme, of BSA and of their mixture as a function of protein concentration between 0 and 40 g/L at pH 7.0 in 1.0 M ionic-strength ammonium-sulfate solution. The osmotic second virial coefficients for lysozyme and BSA are obtained from the slope of a plot of $\Pi/c_i RT$ versus c_i, where **i** stands for lysozyme, for BSA or for the mixture. For c_i between 0 and 40 g/L, the slope is constant. The inverse of the intercept is the molecular weight at infinite dilution.

Table 2 presents number-average molecular weights, osmotic second virial coefficients (B_{22}, B_{33} and B_{23}) and their standard deviations for lysozyme, for BSA and for their mixture.

Truncated Virial Equation of State

To calculate the potential of mean force of equation (1) for the aqueous saline protein solution, physicochemical properties of the protein are required. These properties include

molecular weight, protein charge (as a function of pH) and hydrodynamic radius, and the charges and sizes of the salt ions. Table 2 shows the parameters required for calculating osmotic second virial coefficients.

Figure 2 shows experimental and calculated values of $\mathbf{B_{22}}$ as a function of pH. The osmotic second virial coefficient of lysozyme is slightly negative under all conditions studied, indicating that the net interactions between aqueous lysozyme molecules are attractive. The osmotic second virial coefficient becomes more negative as pH or ionic strength rises. For calculating the potential of mean force for lysozyme, the Hamaker constant was set equal to 7.0 kT. Experimental values of $\mathbf{B_{22}}$ are in reasonable agreement with those calculated $\mathbf{B_{22}}$ at 1.0 M ionic strength. However, the calculated values at 3.0 M ionic strength are more negative than those measured. The effect of ionic strength on the potential of mean force is included in the model through the osmotic-attraction potential, which is a first approximation for the indirect effect of the excluded volume of the ions on the effective protein-protein interaction. In this potential, when the distance between two protein surfaces is less than the mean salt diameter, the salt ions are squeezed out from between two protein surfaces. This results in an attraction due to the difference in osmotic pressure of the salt outside of the protein molecules versus that between the protein molecules. In this highly approximate osmotic potential, the osmotic pressure of the salt is given by the ideal Van't Hoff law and the excluded volume of the water molecules is neglected although the size of the water molecule is similar to that of an ion. Thus, accurate predictions of the potential of mean force model are not expected because the osmotic attraction potential used here gives an over-simplified description of protein-protein attraction in a concentrated electrolyte solution.

Figure 3 shows experimental and calculated values of $\mathbf{B_{33}}$ as a function of pH. The osmotic second virial coefficient of BSA is positive for all experimental conditions, indicating a net repulsion between aqueous BSA molecules. The osmotic second virial coefficient increases with rising pH and falls slightly with rising ionic strength. For calculating the potential of mean force for BSA, the Hamaker constant was set equal to 3.0 kT. The experimental data show that as pH rises, the intermolecular attraction decreases slightly. This is not predicted by the potential of mean force model where the pH dependence is taken into account by changing the electric double-layer repulsion by varying the net charge of the protein. However, the contribution of the double-layer repulsion is negligible at 1.0 M ionic strength. As ionic strength increases from 1.0 M to 3.0 M, there is a very small decrease in the observed $\mathbf{B_{33}}$. This result is qualitatively opposite to that for lysozyme and is not predicted by the osmotic-attraction potential.

Using equation (7), we calculated the osmotic second virial cross coefficient, $\mathbf{B_{23}}$, for the mixture. Figure 4 shows experimental and calculated value of $\mathbf{B_{23}}$. At 1.0 M ionic strength, it becomes slightly positive with rising pH and at 3.0 M ionic strength it becomes slightly negative with rising pH. For calculating $\mathbf{B_{23}}$, the Hamaker constant for the cross interaction was set equal to 3.0 kT. Our calculated results using the truncated virial equation of state indicate that the intermolecular forces for an aqueous lysozyme-BSA pair are intermediate between those for the lysozyme-lysozyme and BSA-BSA pairs.

Concentrated Protein Solution: RPA Model

The truncated virial equation is valid only for low protein concentrations, below (about) 20 or 40 g/L. For higher protein concentrations, we use the random-phase-approximation (RPA) model. Figure 5 compares experimental osmotic-pressure data with those calculated using RPA theory for lysozyme, for BSA and for the mixture in 1.0 M ionic-strength ammonium-sulfate solution. Fitted osmotic pressures are in reasonable agreement with experiment below protein concentrations of 30 g/L; at higher concentrations, calculated and observed results disagree.

Table 4 shows Hamaker constants for lysozyme, for BSA, and for the mixture obtained from fitting the data below 40 g/L to RPA theory. Reduced Hamaker constants range from 24.9 to 38.8 for lysozyme and from 4.0 to 12.5 for BSA. These values are significantly larger than those calculated from fitting the data to the truncated virial equation of state in the dilute protein concentration region, where only two-body interactions are important and where the virial equation of state is valid. Thus, we expect that the RPA calculation of the osmotic pressure is in error because it is based on inappropriate simplifying assumptions. It is likely that the error is in the perturbation term of the RPA, because the Carnahan Starling equation for the reference system is believed to be accurate over the entire concentration range. This conclusion can be demonstrated by comparing the reference terms and the perturbation terms for the osmotic pressure from the RPA and from the truncated virial equation of state.

The hard-sphere reference contribution to the truncated virial equation of state is given by

$$Z_{hs} = 1 + B'_{22}\rho = 1 + 4\eta \qquad (20)$$

where

$$B'_{hs} = \frac{2}{3}\pi\sigma^3 \qquad (21)$$

The reference term of the RPA model is given by equation (11). Figure 6 shows the contribution of the reference term to the osmotic pressure as a function of protein concentration. The reference-term contributions are comparable for the two models for dilute concentration. As expected, the truncated virial equation of state does not agree with the results of the Carnahan-Starling equation at high concentrations because in the former, only two-body hard-sphere interactions are included. For better accuracy at higher concentrations, higher order hard-sphere virial coefficients need to be included in the virial equation of state.

Figure 7 shows the contribution of the perturbation term calculated using the truncated virial equation of state and using the RPA from the potential of mean force without the osmotic attraction term. Perturbation terms are plotted over a range of 0 to 40 g/L, where the reference terms of the virial equation of state and RPA are essentially identical. As shown in Figure 7, contributions to the osmotic pressure from RPA differ from those contributed by the truncated virial equation of state for Hamaker constants greater than 5.0 kT. The virial equation of state should be accurate for the low protein concentration range of 0 to 40 g/L. In this range, the long-ranged correlation between molecules is determined from the two-body perturbation interactions. In the RPA, this correlation is neglected. As the interaction strength increases, the correlation becomes more significant and the prediction of the RPA is in error.

Concentrated Protein Solution: AHS Model

The adhesive hard-sphere model was used to correlate the experimental osmotic pressure in the concentrated protein region where the truncated virial equation of state is not applicable and where the random-phase approximation is in error. Figures 8 and 9 show experimental and calculated osmotic pressures for lysozyme, for BSA, and for the lysozyme-BSA mixture at pH 7.0 in 1.0 M and 3.0 M ionic-strength ammonium-sulfate solution, respectively, using τ as the only fitting parameter. For the mixture, τ_{23} was fit from the mixture data using the values of τ_{22} and τ_{33} determined from fitting the pure-component osmotic-pressure data. The fit of the osmotic-pressure data indicates that the adhesive hard-sphere potential provides a semi-accurate description of the protein-protein interactions in concentrated protein solutions. The forces between proteins in concentrated salt solution are indirect, that is, they are the result of many-body forces; they are poorly understood. Because the adhesion potential provides a good effective description of these interactions, it appears that the solvation forces are short-ranged in concentrated salt solutions, as indicated by the work of Piazza (1999) and Rosenbaum et al., (1996), where it is shown that protein-protein interactions under crystallization conditions are short-ranged.

Figure 10 shows the reciprocal of τ which is proportional to the strength of intermolecular attraction. All values of inverse τ for BSA are very small, indicating that the interaction between BSA molecules is determined primarily by the hard-sphere repulsion term. However, the interactions between lysozyme molecules are slightly attractive and the attraction increases with raising ionic strength. The interactions between lysozyme and BSA are intermediate between those for lysozyme-lysozyme and

those for BSA-BSA interactions. Analysis of the second-virial-coefficient data provided the same conclusions concerning the overall potential of mean force. However, the main difference between analyzing $\mathbf{B}_{ij}$ versus evaluating inverse τ is that the values of inverse τ provide a measure of the attractive forces alone where the hard-sphere repulsion has been subtracted out.

In conclusion, we have measured the interactions between lysozyme-lysozyme, BSA-BSA, and lysozyme-BSA pairs. The interactions between lysozyme molecules are slightly attractive, while the interactions between BSA pairs are dominated by the hard-sphere repulsion terms. The attractive part of the cross interaction is inbetween those of the pure interactions. A simple uniform adhesive hard-sphere potential model provides a fair fit to the data, indicating that the attractive interactions are short-ranged.

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Literature Cited

Asakura, S. and F. Oosawa, "Interaction Between Particles Suspended in Solutions of Macromolecules", J. Polym. Sci., Vol. **33**, 183 (1958).

Barboy, B., "On a Representation of the Equation of State of Fluids in Terms of the Adhesive Hard-Sphere Model", J. Chem. Phys., Vol. **61**, No. **8**, 3194 (1974).

Barboy, B., R. Tenne, "Distribution Functions and Equation of State of Sticky Hard Sphere Fluids in the Percus-Yevick Approximation", Chem. Phys., Vol. **38**, 369 (1979).

Baxter, R. J., "Percus-Yevick Equation for Hard Spheres with Surface Adhesion", J. Chem. Phys., Vol. **49**, No. **6**, 2770 (1968).

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

Carnahan, N. F. and K. E. Starling, "Equation of State for Nonattracting Rigid Spheres", J. Chem. Phys., Vol. **51**, No. **2**, 635 (1969).

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

Chiew, Y. C., D. Kuehner, H. W. Blanch and J. M. Prausnitz, "Molecular Thermodynamics for Salt-induced Protein Precipitation", AIChE J., **41**, 2150 (1995).

Curtis, R. A., J. M. Prausnitz, H. W. Blanch, "Protein-Protein and Protein-Salt Interactions in Aqueous Protein Solutions Containing Concentrated Electrolytes", Biotech and Bioeng., Vol. **57**, No. **1**, 11 (1998).

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

Grimson, M. J., "Small Angle Scattering from Colloidal Solutions", J. Chem. Soc. Farad. Trans. II, Vol. **79**, 817 (1983).

Israelachvili, J. N., "Intermolecular and Surface Forces: with Applications to Colloidal and Biological Systems., 2nd ed., Academic Press, London, (1992).

Kuehner, D. E., C. Heyer, C. Ramsch, U. M. Fornefeld, H. W. Blanch, and J. M. Prausnitz, "Interactions of Lysozyme in Concentrated Electrolyte Solutions from Dynamic Light-Scattering Measurements", Biophys. J., Vol. **73**, 3211 (1997).

Kurata, M., " Thermodynamics of Polymer Solutions", Harwood Academic Publishers Vol. **1**, 51 (1982).

Mansoori, G. A. et al., "Equilibrium Thermodynamic Properties of the Mixture of Hard Sphere", J. Chem. Phys., Vol. **54**, 1523 (1971).

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

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

Pizza R., "Interactions in Protein Solutions near Crystallization: A Colloid Physics Approach", J. Crystal Growth, Vol. **196**, 415 (1999).

Rosenbaum, D. F., P. C. Zamora, and C. F. Zukoski, "Phase Behavior of Small Attractive Colloidal Particles", J. Phys. Review Letters, Vol. **183**, 452 (1996).

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

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

Vilker, V. L., C. K. Colton, and K. A. Smith, "The Osmotic Pressure of Concentrated Protein Solution: Effect of Concentration and pH in Saline Solution of Bovine Serum Albumin", J. Colloid and Interface Sci., Vol. **79**, No. **2**, 548 (1981).

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

Table 1. Experimental Osmotic-Pressure Data for Lysozyme, BSA and for the Mixture in 1.0 M and 3.0 M Ionic Strength $(NH_4)_2SO_4$ Salt Solution.

	Protein Concentration (g/L)			Weight Fraction*		Osmotic Pressure (mmHg)		
	Lysozyme	BSA	Mixture**	w_2	w_3	Lysozyme	BSA	Mixture***
	10.21	8.70	9.46	0.54	0.46	11.5	2.2	6.6
	20.35	17.12	18.74	0.54	0.46	22.5	4.4	13.0
IS = 1.0 M	30.80	26.82	28.81	0.53	0.47	33.5	6.9	19.9
pH 4	40.44	35.45	37.95	0.53	0.47	43.2	9.2	26.1
	60.55	53.16	56.86	0.53	0.47	62.4	14.0	39.0
	81.09	70.55	75.82	0.53	0.47	80.8	19.0	51.5
	100.00	88.24	94.12	0.53	0.47	97.0	24.4	63.5
	9.98	9.72	9.85	0.51	0.49	10.7	2.6	6.8
	20.31	19.06	19.69	0.52	0.48	21.1	5.3	13.5
IS = 1.0 M	30.62	28.42	29.52	0.52	0.48	30.8	8.1	20.2
pH 6	40.94	37.74	39.34	0.52	0.48	40.0	11.3	26.9
	60.73	56.40	58.56	0.52	0.48	55.9	19.0	39.7
	80.56	74.63	77.59	0.52	0.48	70.8	27.9	52.3
	100.49	92.67	96.58	0.52	0.48	85.7	38.8	64.6
	10.33	9.68	10.01	0.52	0.48	11.0	2.6	7.0
	19.79	19.51	19.65	0.50	0.50	20.7	5.6	13.6
IS = 1.0 M	30.66	29.74	30.20	0.51	0.49	30.7	8.9	20.9
pH 7	40.95	39.28	40.12	0.51	0.49	39.6	12.4	27.7
	60.83	58.89	59.86	0.51	0.49	55.0	20.6	40.9
	80.76	77.77	79.26	0.51	0.49	70.9	31.6	54.0
	101.97	96.83	99.40	0.51	0.49	83.6	46.5	68.0
	10.14	9.80	9.97	0.51	0.49	10.6	2.7	7.0
	20.06	19.38	19.72	0.51	0.49	20.0	5.6	13.7
IS = 1.0 M	30.18	28.85	29.51	0.51	0.49	28.5	8.9	20.3
pH 8	40.14	38.69	39.42	0.51	0.49	36.3	12.6	26.8
	50.37	47.83	49.10	0.51	0.49	43.6	16.6	32.7
	59.42	57.21	58.31	0.51	0.49	51.5	21.1	38.7
	4.80	19.82	-	-	-	5.4	5.0	-
IS = 3.0 M	7.18	27.69	-	-	-	8.0	7.0	-
pH 4	9.65	36.45	-	-	-	10.5	9.2	-
	14.36	55.12	-	-	-	15.4	14.0	-
	19.13	72.90	-	-	-	19.8	18.6	-
	10.29	9.64	9.97	0.52	0.48	10.6	2.5	6.7
IS = 3.0 M	15.44	14.45	14.95	0.52	0.48	15.4	3.8	9.9
pH 6	20.79	19.15	19.97	0.52	0.48	20.1	5.1	13.0
	25.80	24.05	24.92	0.52	0.48	24.1	6.5	16.1
	30.74	28.69	29.71	0.52	0.48	27.7	7.9	19.2
	9.36	10.05	9.71	0.48	0.52	9.7	2.6	6.3
IS = 3.0 M	12.62	13.46	13.04	0.48	0.52	12.9	3.6	8.4
pH 7	18.86	20.03	19.44	0.49	0.51	18.4	5.4	12.3
	25.14	26.80	25.97	0.48	0.52	23.4	7.4	16.3
	31.42	33.59	32.50	0.48	0.52	26.8	9.5	19.8
	9.92	9.21	9.57	0.52	0.48	10.1	2.4	6.4
IS = 3.0 M	14.86	13.82	14.34	0.52	0.48	14.5	3.7	9.4
pH 8	19.85	18.48	19.16	0.52	0.48	18.5	5.1	12.4
	24.74	23.01	23.87	0.52	0.48	22.0	6.4	15.2
	29.59	27.56	28.57	0.52	0.48	25.0	7.8	17.9

* Weight Fraction for the Mixture (w_1 or w_2) = (c_2 or c_3 /2)/c_T, c_2 (g/L); Lysozyme, c_3 (g/L); BSA.

** Total Protein Concentration for the Mixture (c_T) = [c_2 + c_3]

*** Measured Osmotic Pressure between 10 - 100 and 10 - 30 g/L for 1.0 and 3.0 M Ionic Strength.

Table 2. Measured $\mathbf{B_{22}}$, $\mathbf{B_{33}}$, $\mathbf{M_{n2}}$, and $\mathbf{M_{n3}}$ for Lysozyme and BSA, and $\mathbf{B_{23}}$ for the Mixture in Ammonium Sulfate Solution at 25°C*

		Lysozyme		BSA		Mixture
IS (M)	pH	$\mathbf{B_{22}}$ (10^{-4} mol-ml/g^2)	$\mathbf{M_{n2}}$ (g/mol)	$\mathbf{B_{33}}$ (10^{-4} mol-ml/g^2)	$\mathbf{M_{n3}}$ (g/mol)	$\mathbf{B_{23}}$ (10^{-4} mol-ml/g^2)
1.0	4.0	-1.01 ± 0.02	16,322	0.12 ± 0.03	74,269	-0.37 ± 0.05
	6.0	-1.65 ± 0.04	16,964	0.59 ± 0.06	72,945	0.38 ± 0.15
	7.0	-1.76 ± 0.15	16,938	0.83 ± 0.05	73,407	0.23 ± 0.29
	8.0	-2.68 ± 0.22	16,975	0.94 ± 0.05	72,732	0.14 ± 0.23
3.0	4.0	-3.30 ± 0.55	16,299	0.03 ± 0.01	74,392	-
	6.0	-3.27 ± 0.06	17,106	0.41 ± 0.01	74,191	-0.37 ± 0.40
	7.0	-3.66 ± 0.19	16,930	0.49 ± 0.12	74,204	-0.22 ± 0.43
	8.0	-4.72 ± 0.22	16,882	0.71 ± 0.04	75,044	-0.31 ± 0.33

* Experimental Data were used between 0 to 40 and 0 to 25 g/L of Protein Solution in 1.0 and 3.0 M IS, respectively.

Table 3. Net Charge and Ion Size Parameters for Calculating Potential of Mean Force

	pH	Lysozyme	BSA
Net Charge*	4.0	14.0	20.0
	6.0	9.0	-13.5
	7.0	8.0	-18.8
	8.0	7.5	-22.9
Ion Size Parameters** (Diameter, Å)	Lysozyme	34.4	
	BSA	62.6	
	NH_4^+	2.13	
	SO_4^{2-}	2.78	

* Effect of pH on Net Charge in 1.0 M KCl Solution for Lysozyme and 0.15 M NaCl Solution for BSA by Titration Method

** Kuehner, D. E. et al., (1997); Vilker, V. L., et al., (1981); Marcus, Y., (1994)

Table 4. Reduced Hamaker Constants (H/kT) for Lysozyme, for BSA and for the Mixture Using the RPA Model for Data Reduction

IS (M)	pH	Lysozyme	BSA	Mixture
1.0	4.0	24.9	12.0	2.8
	6.0	29.2	7.4	2.8
	7.0	29.3	5.4	2.6
	8.0	33.0	4.0	2.7
3.0	4.0	36.3	12.5	-
	6.0	34.9	8.9	3.3
	7.0	37.4	7.7	3.5
	8.0	38.8	7.7	3.7

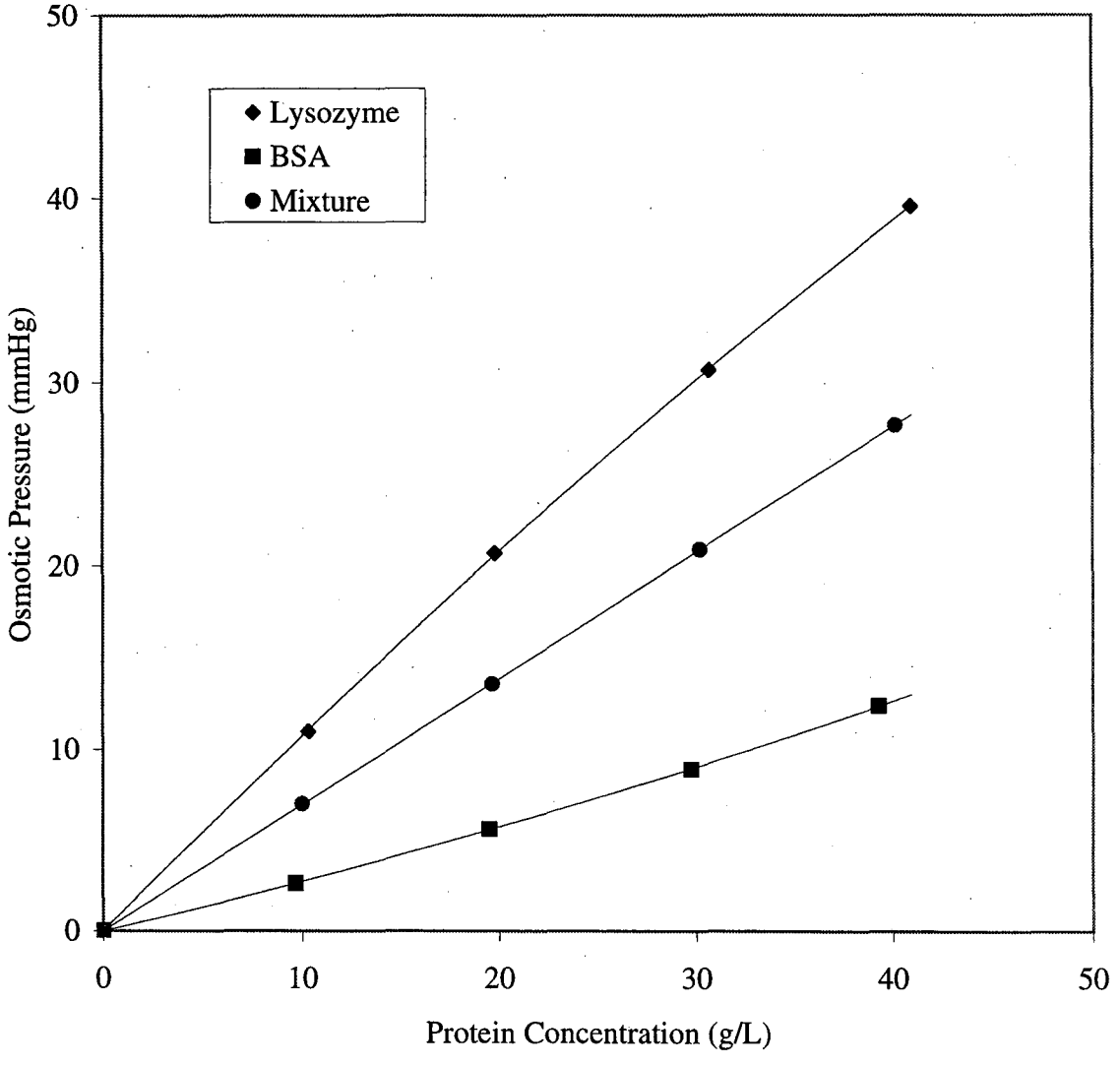

Fig. 1. Osmotic Pressures for Lysozyme, for BSA and for the 1:1 Volume Mixture at pH 7 in 1.0 M Ionic Strength $(NH_4)_2SO_4$ Solution

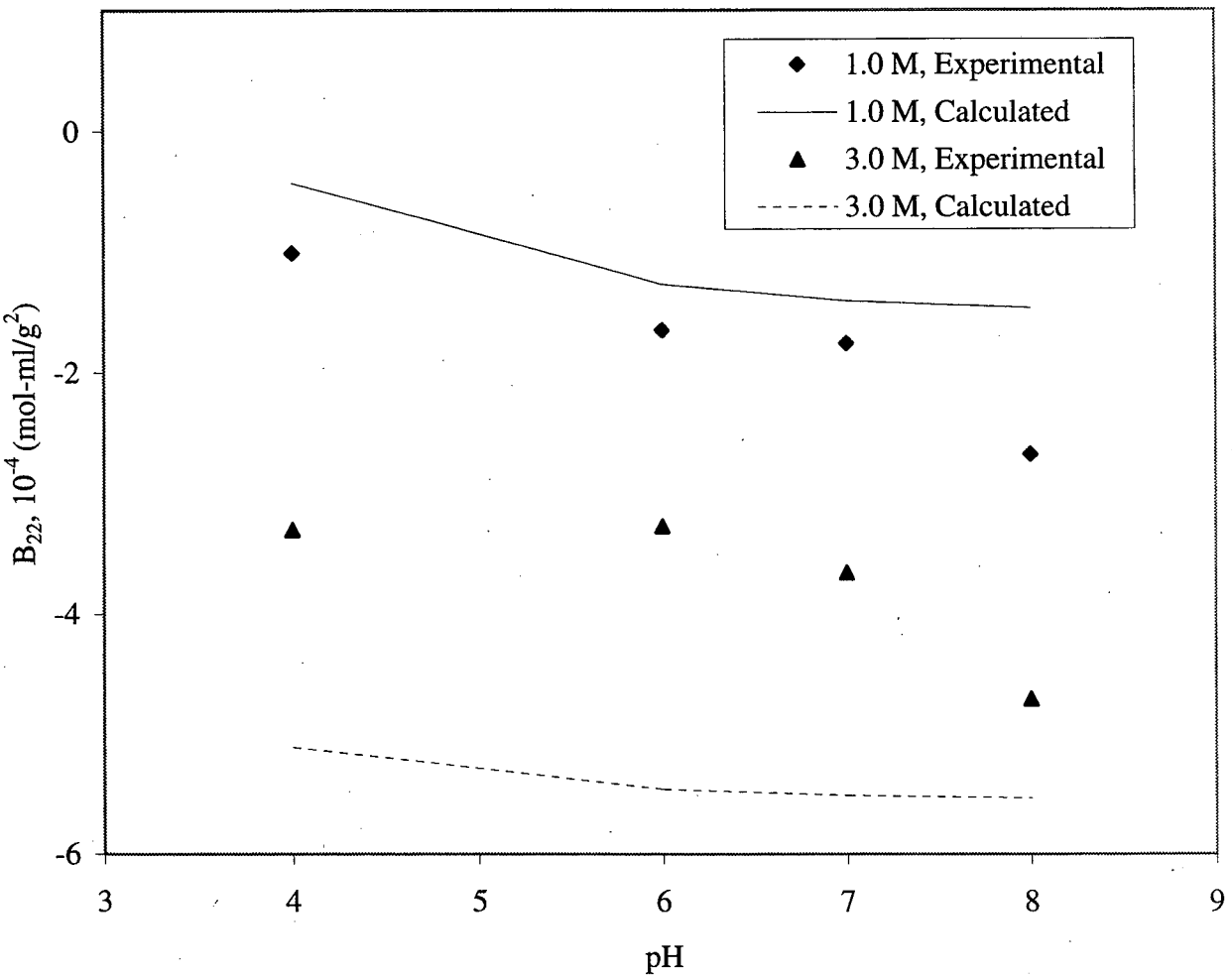

Fig. 2. Experimental and Calculated Osmotic Second Virial Coefficient B_{22} vs. Solution pH for Lysozyme in Ammonium-Sulfate Solution. Calculations Based on Truncated Virial Equation with H = 7.0 kT.

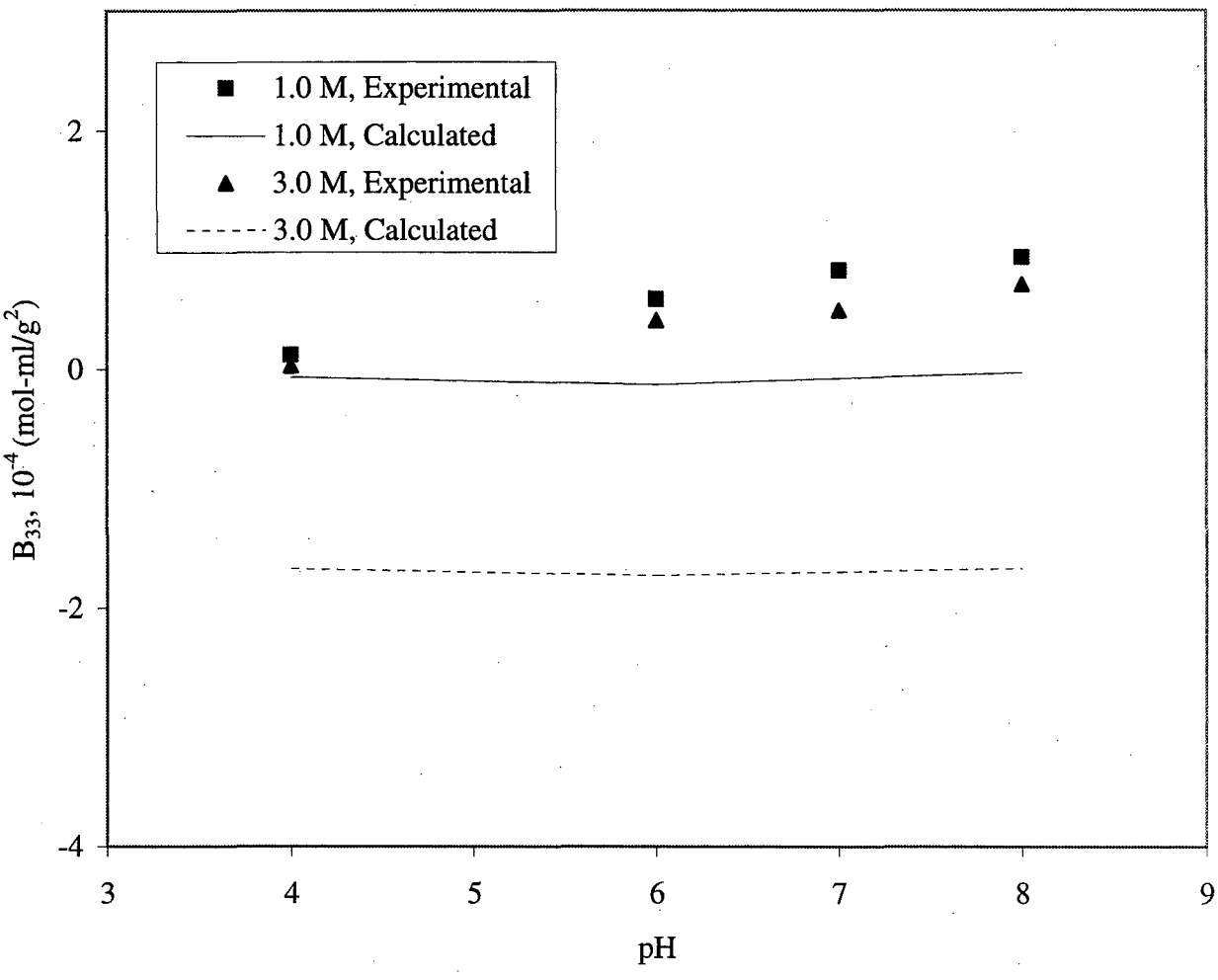

Fig. 3. Experimental and Calculated Osmotic Second Virial Coefficient B_{33} vs. Solution pH for BSA in Ammonium-Sulfate Solution. Calculations Based on Truncated Virial Equation with H = 3.0 kT.

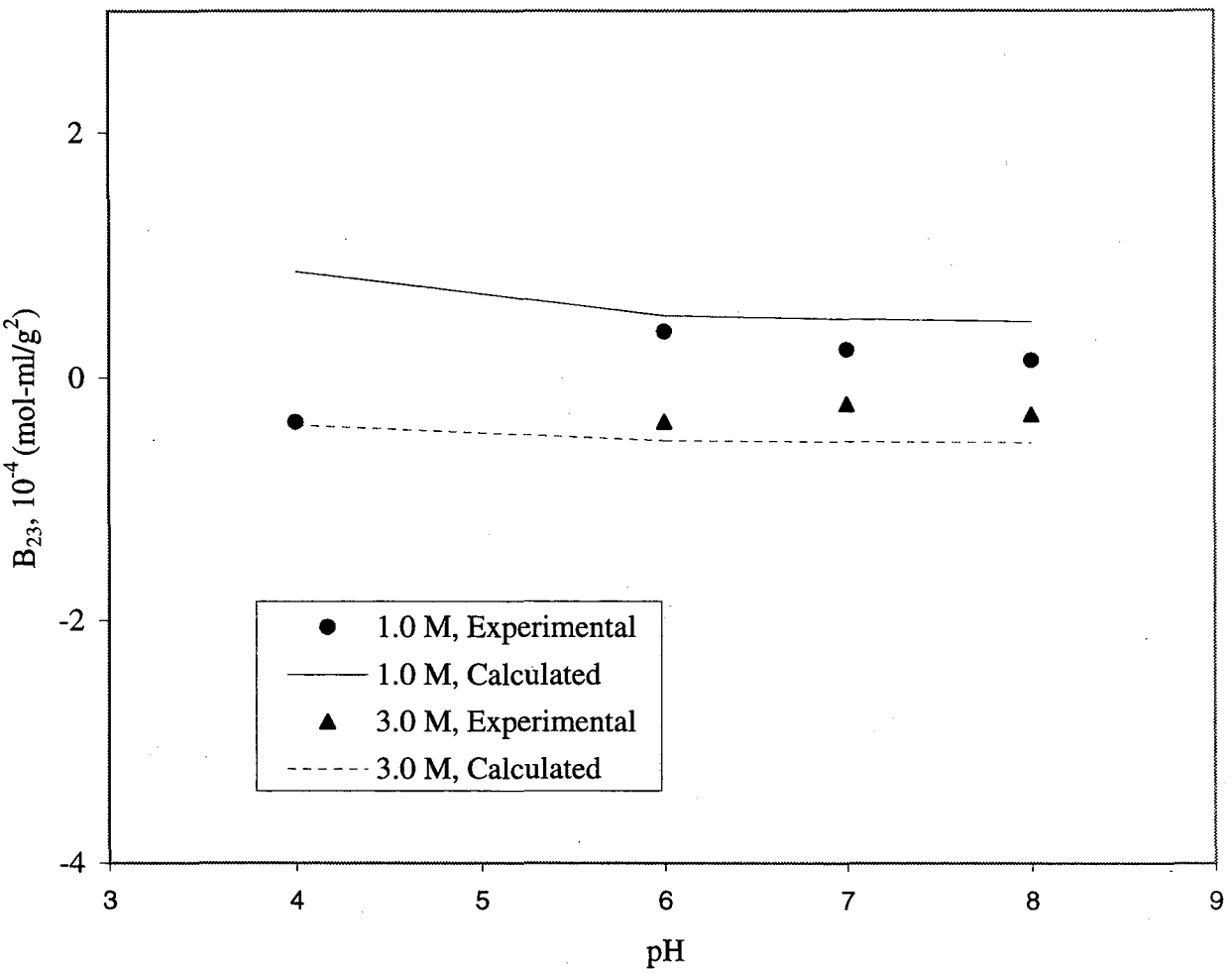

Fig. 4. Experimental and Calculated Osmotic Second Virial Coefficient B_{23} vs. Solution pH for the Mixture in Ammonium-Sulfate Solution. Calculations Based on Truncated Virial Equation with H = 3.0 kT.

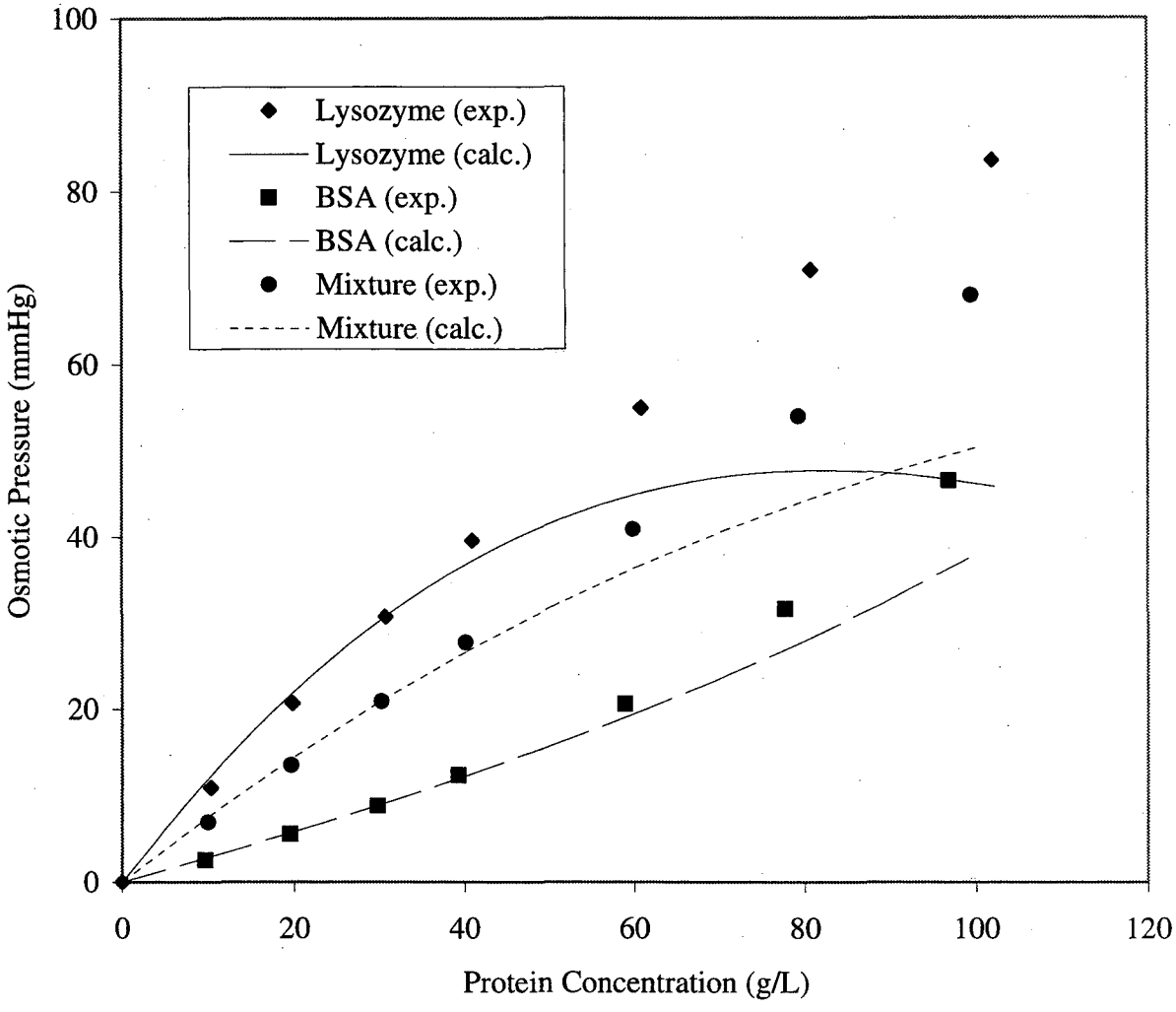

Fig. 5. Experimental and Calculated (RPA) Osmotic Pressures for Lysozyme, for BSA and for the Mixture in 1.0 M Ionic Strength Ammonium Sulfate Solution at pH 7.

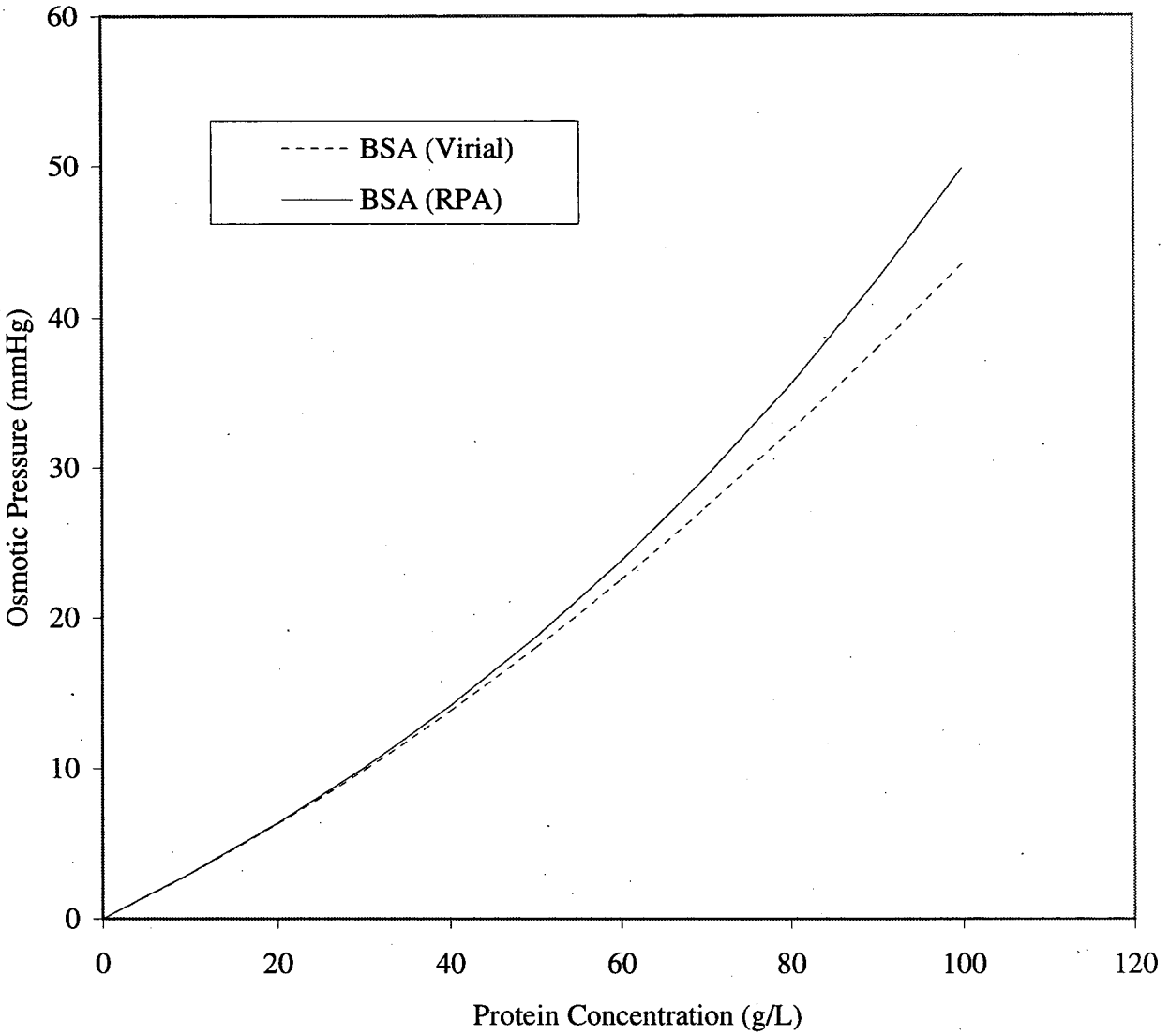

Fig. 6. Contribution to Osmotic Pressure of Reference Term Using Truncated Virial Equation of State and RPA Model for Lysozyme and for BSA with Structured Water Layer=1.0 Å Added to the Protein Radius.

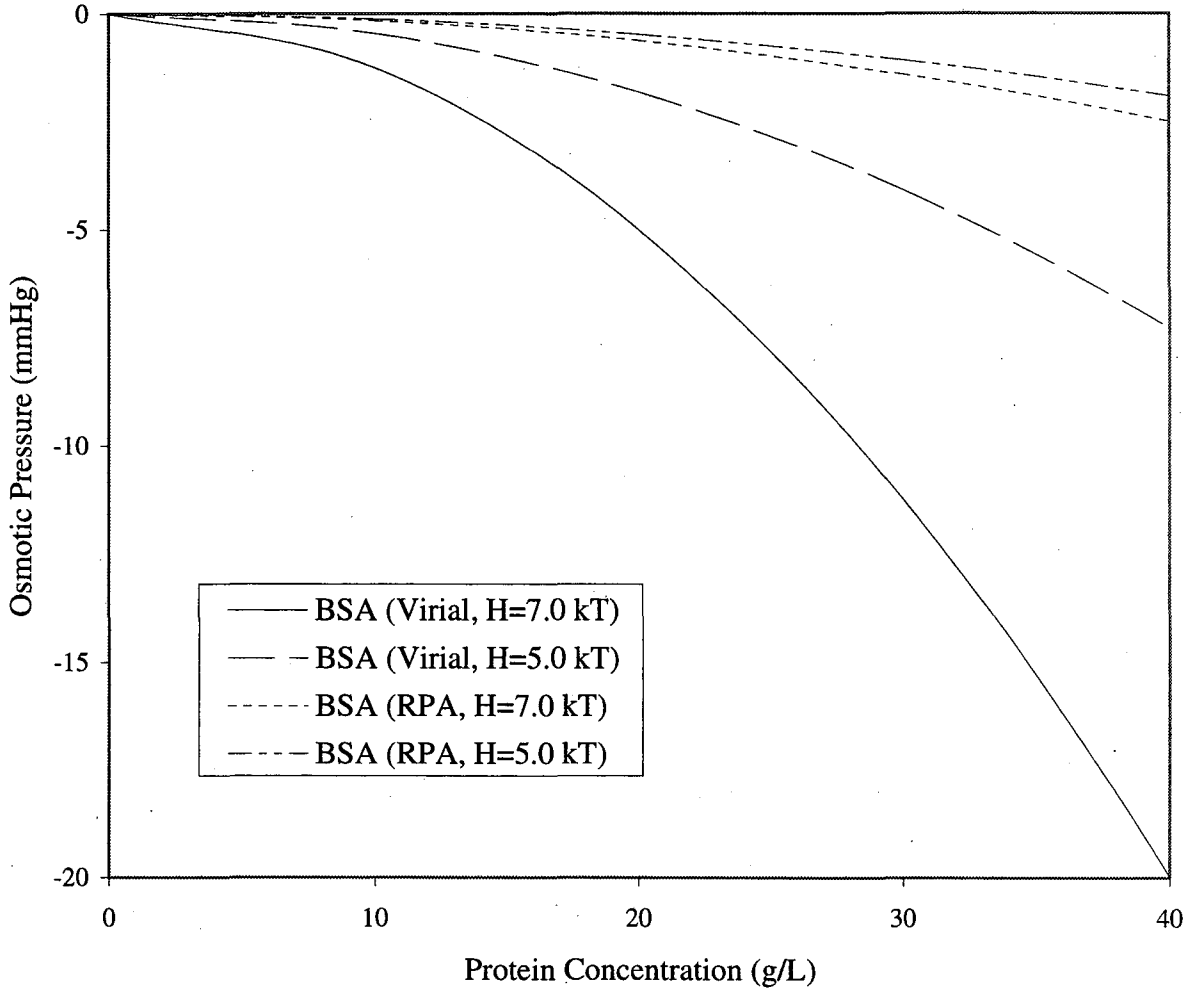

Fig. 7. Contribution to Osmotic Pressure of Perturbation Term Using Truncated Virial Equation of State and RPA Model for BSA for Two Values of Reduced Hamaker Constant.

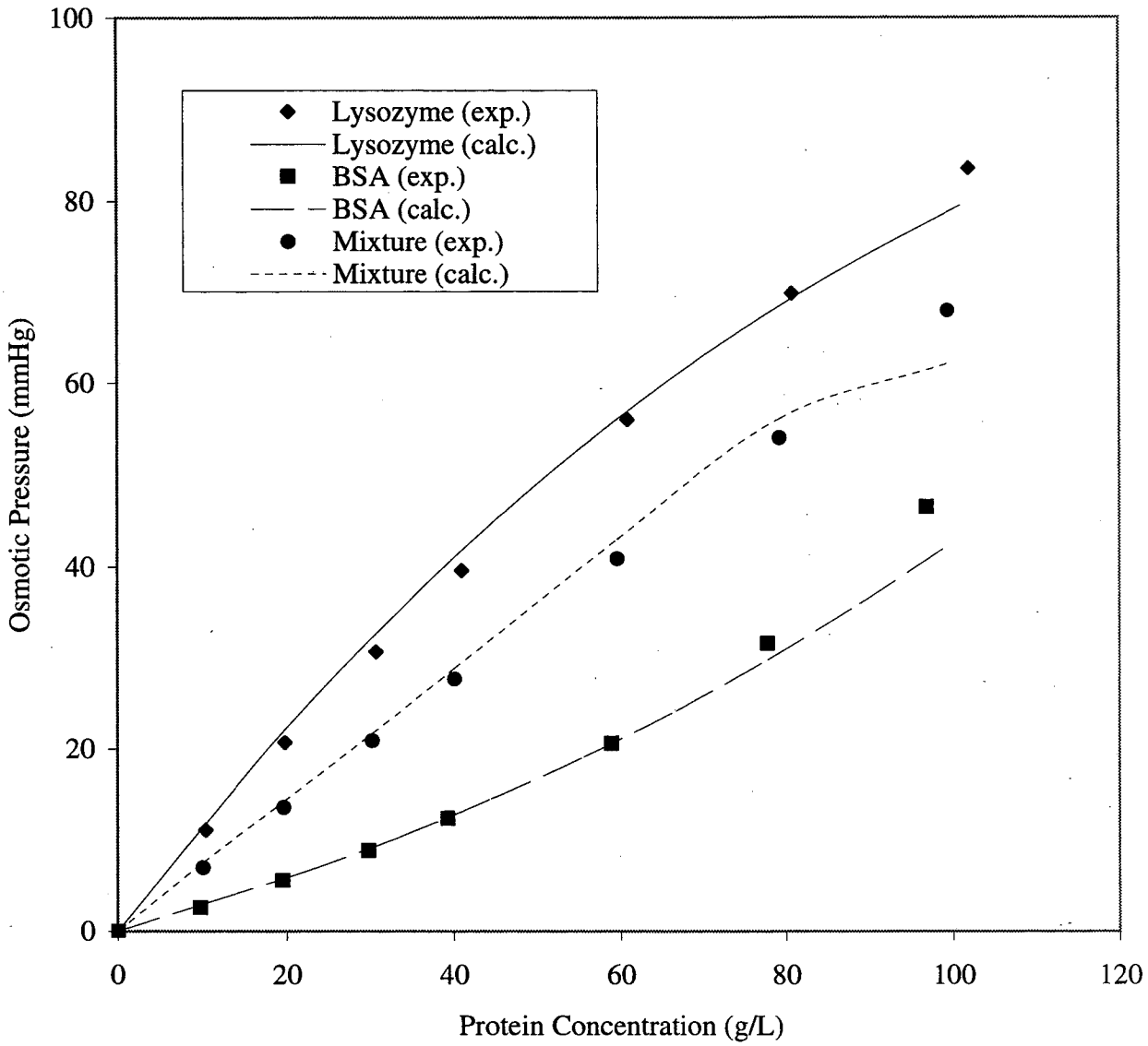

Fig. 8. Experimental and Calculated (AHS) Osmotic Pressures for Lysozyme, BSA, and the Mixture in 1.0 M Ionic-Strength Ammonium-Sulfate Solution at pH 7.

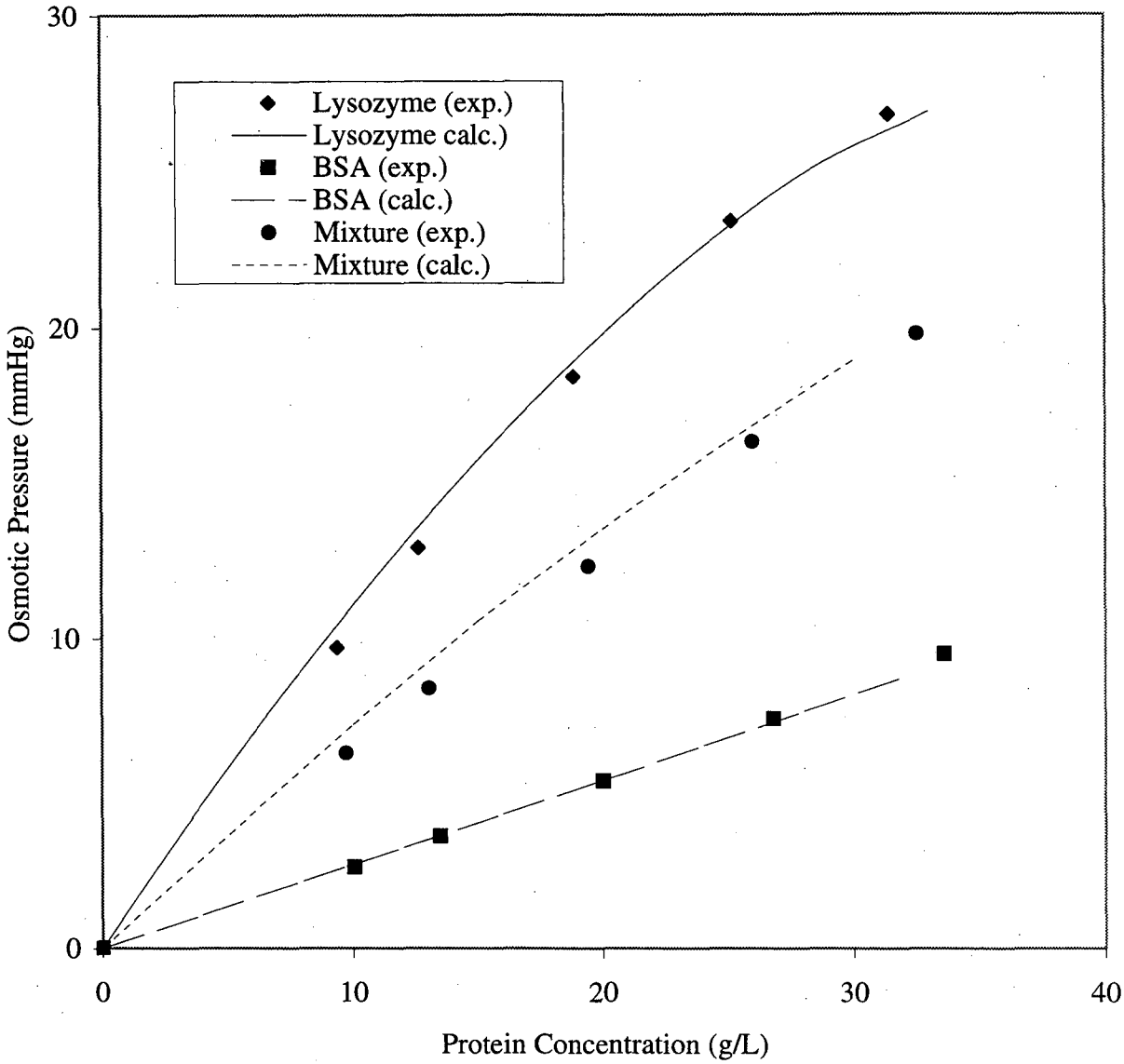

Fig. 9. Experimental and Calculated (AHS) Osmotic Pressures for Lysozyme, BSA, and the Mixture in 3.0 M Ionic-Strength Ammonium-Sulfate Solution at pH 7.

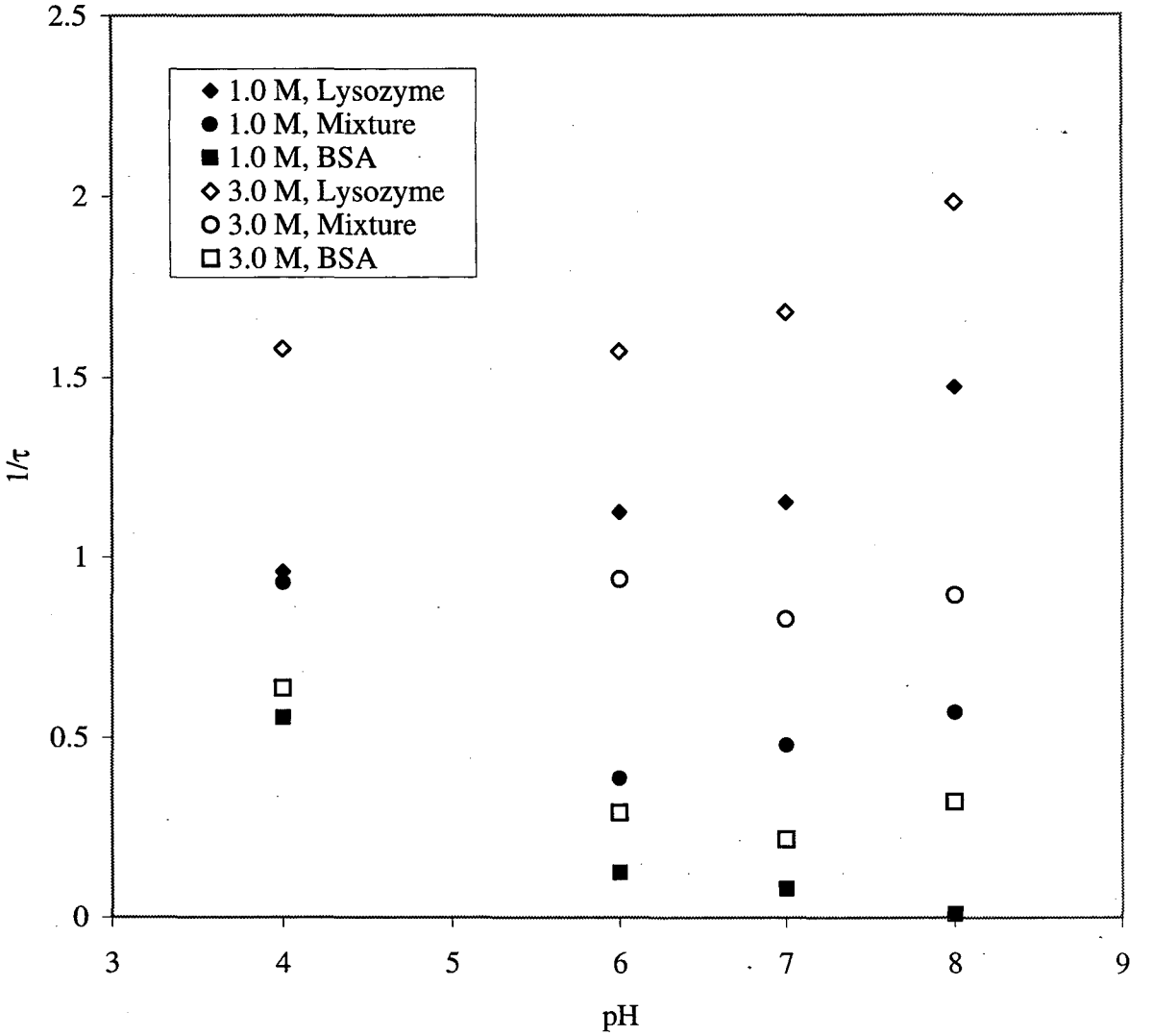

Fig. 10. Attraction Parameters in AHS Model for Lysozyme, BSA, and the Mixture as a Function of pH and Ionic Strength.

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