Extraction of Thermodynamic Data from Ternary Diffusion Coefficients. Use of Precision Diffusion Measurements for Aqueous Lysozyme Chloride−NaCl at 25 °C To Determine the Change of Lysozyme Chloride Chemical Potential with Increasing NaCl Concentration Well into the Supersaturated Region†

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Abstract: For ternary systems, we present a method for using measured values of the four ternary diffusion coefficients and the Onsager reciprocal relations to extract derivatives of solute chemical potentials with respect to solute molar concentrations. The method is applicable to systems in which the molar concentration of one solute is very small compared to that of the other, and also small enough that an inverse concentration dependence dominates certain activity coefficient derivatives. These conditions apply to a large number of aqueous systems involving macromolecules of biological interest. Unlike other techniques, the present method can be used to study undersaturated and supersaturated solutions. The approach is illustrated for the lysozyme chloride−NaCl−H_2O system at 25 °C, using data reported here for pH 6.0 at 0.60 mM (8.6 mg/mL) lysozyme chloride and 0.25, 0.50, 0.65, 0.90, and 1.30 M (1.4, 2.8, 3.7, 5.1, and 7.2 wt %) NaCl concentrations, and our earlier data for pH 4.5 at the same concentrations. We use these solute chemical potential derivatives to compute the protein cation charge approximately, and to construct a function approximating the derivative of the lysozyme chloride chemical potential with respect to NaCl concentration, which we integrate over a range of NaCl concentrations. This provides the change of the lysozyme chloride chemical potential with NaCl concentration well into the supersaturated region, and hence provides the driving force for nucleation and crystal growth of lysozyme chloride as a function of the extent of supersaturation. We also compute the diffusion Onsager coefficients $(L_{ij})_0$ for each composition at pH 4.5 and 6.0. Binary diffusion coefficients of aqueous lysozyme chloride at 0.89 mM (12.7 mg/mL) for pH values from 4.0 to 6.0, and at pH 6.0 for concentrations from 0.25 to 1.95 mM (3.6−27.9 mg/mL) are also reported.

Introduction

Thermodynamic properties are typically determined from measurements in equilibrium or quasi-equilibrium experiments. However, that need not be the case. For example, binary diffusion coefficient measurements in dilute solutions have long been used to compute activity coefficients.[1] The use of ternary diffusion coefficients to determine binding coefficients and other thermodynamic data is also well established.[2−4]

Here, we consider a class of ternary systems in which the molar concentration of one solute is very small compared to that of the other, and also small enough that an inverse concentration dependence dominates certain activity coefficient derivatives. For such systems, we show how the Onsager reciprocal relations (ORR), along with precision measurements of ternary diffusion coefficients, can be used to determine concentration derivatives of the chemical potentials of two solutes with respect to solute molar concentrations. The approach is illustrated for lysozyme chloride in aqueous NaCl.

Lysozyme chloride−H_2O and lysozyme chloride−NaCl−H_2O systems serve as models in a wide range of protein studies (e.g., crystal growth) involving kinetic, transport, and equilibrium processes, as discussed in ref 5. Multicomponent diffusion is important in many protein phenomena, including modeling of diffusion to the surface of a growing crystal. Diffusive transport is especially important under microgravity conditions, where buoyancy-driven convective transport is greatly reduced. Other

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(1) Harned, H. S. *Proc. Natl. Acad. Sci. U.S.A.* **1954**, *40*, 551−556.

(2) Paduano, L.; Sartorio, R.; Vitagliano, V.; Albright, J. G.; Miller, D. G.; Mitchell, J. *J. Phys. Chem.* **1990**, *94*, 6885−6888.

(3) Paduano, L.; Sartorio, R.; Vitagliano, V.; Costantino, L. *Ber. Bunsen-Ges. Phys. Chem.* **1990**, *94*, 741−745.

(4) Kim, H. *J. Solution Chem.* **1974**, *3*, 271−287.

(5) Albright, J. G.; Annunziata, O.; Miller, D. G.; Paduano, L.; Pearlstein, A. J. *J. Am. Chem. Soc.* **1999**, *121*, 3256−3266.

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than the work of Leaist and Hao[6−8] in dilute solution and our recent work[5] at higher concentrations relevant to crystal growth, we are unaware of reliable measurements of multicomponent diffusion coefficients in protein systems.[9] However, virtually all studies of protein diffusion are conducted in systems with two or more solutes, and pseudobinary diffusion coefficients for the protein are insufficient to describe the actual protein transport. This lack of measured multicomponent diffusion coefficients for protein systems motivates our systematic program to measure, interpret, and ultimately predict these important transport properties. Such data are critical to modeling of protein crystal growth.[5]

In an earlier paper,[5] we reported precision interferometric measurements of binary diffusion coefficients for low to moderate concentrations of aqueous lysozyme chloride at 25 °C and pH 4.5, as well as the four elements of the diffusion coefficient matrix for 0.60 mM (8.6 mg/mL) solutions of lysozyme chloride in 0.25−1.30 M (1.4−7.2 wt %) aqueous NaCl. At the higher NaCl concentrations, compositions extended well into the region in which the solution is supersaturated with respect to lysozyme chloride. In this paper, we report similar results for this system at pH 6.0. We also report binary diffusion coefficients of aqueous lysozyme chloride at 0.89 mM (12.7 mg/mL) as a function of pH.

We now outline how our earlier data[5] and these additional data at pH 6.0, all at one fixed lysozyme chloride concentration and covering a range of NaCl concentrations, can be used to obtain the chemical potential derivatives, $\mu_{ij} \equiv \partial\mu_i/\partial C_j$ $(i \neq j)$, from diffusion measurements. (A detailed analysis appears below under the heading Evaluation of the μ_{ij}.) Here, lysozyme chloride and NaCl are denoted as solutes 1 and 2, respectively; C_i is the molarity of solute i; and we have adopted a standard notation used in diffusion studies for the concentration derivatives of the chemical potentials.[10,11] Our procedure is limited to the case where one solute is very dilute in molar terms compared to the other. However, our system and many others of biological interest satisfy this restriction.

In our ternary experiments, the molarity of lysozyme chloride is small compared to that of NaCl. Thus, as we discuss below, the self-derivative for lysozyme chloride, μ_{11}, is dominated by its concentration term, with smaller contributions from the charge and activity coefficient derivative terms. The self-derivative for NaCl, μ_{22}, is essentially that of the binary with minor corrections. As shown below, two additional equations for obtaining the *molarity* cross-derivatives (μ_{12} and μ_{21}, which are unequal[11]) are (a) equality of the *molality* cross-derivatives[12]

$$\frac{\partial\mu_1}{\partial m_2} = \frac{\partial\mu_2}{\partial m_1} \qquad (1)$$

where m_i is the molality of solute i, and (b) the ORR equation

$$\mu_{11}(D_{12})_0 - \mu_{12}(D_{11})_0 = \mu_{22}(D_{21})_0 - \mu_{21}(D_{22})_0 \qquad (2)$$

relating the four molarity derivatives and the ternary diffusion coefficients in a solvent-fixed reference frame $(D_{ij})_0$.[13,14] This approach yields some extremely useful results, including an estimate of the lysozyme charge and a functional approximation to the change of the chemical potential of lysozyme chloride with NaCl concentration. The latter provides the driving force for nucleation and crystal growth of lysozyme chloride as a function of the degree of supersaturation. This, together with the diffusion coefficients, will permit the modeling of protein crystallization processes.

For isothermal diffusion, experiments have thoroughly verified the ORR in dilute[15] and nondilute[11,13,14,16−18] systems. The ORR have also been experimentally verified for linear processes other than isothermal diffusion.[19−22] For isothermal diffusion, activity coefficient derivatives and measured ternary diffusion coefficients are required to verify the ORR. Conversely, we take the ORR to be valid, and use the measured diffusion coefficients to extract the derivatives of the chemical potentials with respect to the concentrations. The significance of this approach is that it allows certain aspects of equilibrium behavior to be predicted using measured transport data and binary thermodynamic data, without resort to approximate theoretical techniques.[11,13,14,23,24]

To the best of our knowledge, the only previous use of the ORR to compute a thermodynamic quantity is the one-dimensional nonequilibrium molecular dynamics calculation of Hafskjold and Ikeshoji[25] for a thermodynamically ideal binary system in which the mass ratio was 10, and the two species had identical molecular diameters and Lennard-Jones intermolecular potential depths. The computed thermal diffusion coefficient and the ORR were then used to evaluate the derivative of the chemical potential of one species with respect to its own mass fraction. Also, to the best of our knowledge, the only previous use of the ORR to extract additional information from experimental data is the work of Hanot et al.,[26] in which the ORR, activity coefficient data, and a measurement of the (Soret) separation ratio were used to compute the off-diagonal diffusion coefficients D_{12} and D_{21} for the ternary system H_2O + ethanol + 2-propanol.

This work differs fundamentally from earlier work in which thermodynamic properties and off-diagonal diffusion coefficients have been related by approximations, rather than the exact ORR. Havenga and Leaist[27] performed ternary diffusion measurements in 50:50 (w/w) water + dioxane (1) + electrolyte (2) solutions for twelve inorganic salts and one organic salt, and used vapor

(6) Leaist, D. G. *J. Phys. Chem.* **1986**, *90*, 6600−6602.
(7) Leaist, D. G. *J. Phys. Chem.* **1989**, *93*, 474−479.
(8) Leaist, D. G.; Hao, L. *J. Chem. Soc., Faraday Trans.* **1993**, *89*, 2775−2782.
(9) In an earlier interferometric study of multicomponent diffusion in protein systems (Ram Mohan, G. Ph.D. Dissertation, Weizmann Institute of Science, Rehovot, Israel, 1965), apparatus quality was poor and data analysis was unsatisfactory. A 1969 conference paper by the same author (Ram Mohan, G. In *Diffusion Processes: Proceedings of the Thomas Graham Memorial Symposium*; Sherwood, J. N., Ed.; Gordon and Breach: London, 1971; pp 139−149) contains no useful information.
(10) Miller, D. G.; Vitagliano, V.; Sartorio, R. *J. Phys. Chem.* **1986**, *90*, 1509−1519.
(11) Miller, D. G. *J. Phys. Chem.* **1959**, *63*, 570−578.
(12) Lewis, G. N.; Randall, M. *Thermodynamics*, 2nd ed.; McGraw-Hill: New York, 1961.
(13) Dunlop, P. J.; Gosting, L. J. *J. Phys. Chem.* **1959**, *63*, 86−93.
(14) Woolf, L. A.; Miller, D. G.; Gosting, L. J. *J. Am. Chem. Soc.* **1962**, *84*, 317−331.
(15) Leaist, D. G.; Lyons, P. A. *Aust. J. Chem.* **1980**, *33*, 1869−1887.
(16) Miller, D. G. *J. Phys. Chem.* **1965**, *69*, 3374−3376.
(17) Spera, F. J.; Trial, A. F. *Science* **1993**, *259*, 204−206.
(18) Käshammer, S.; Weingärtner, H.; Hertz, H. G. *Z. Phys. Chem.* **1994**, *187*, 233−255.
(19) Miller, D. G. *Chem. Rev.* **1960**, *60*, 15−37.
(20) Miller, D. G. In *Transport Phenomena in Fluids*; Hanley, H. J. M., Ed.; Marcel Dekker: New York, 1969; pp 377−427.
(21) Miller, D. G. In *Foundations of Continuum Thermodynamics*; Delgado Domingos, J. J., Nina, M. N. R., Whitelaw, J. H., Eds.; Wiley: New York, 1973; pp 185−214.
(22) Rowley, R. L.; Horne, F. H. *J. Chem. Phys.* **1978**, *68*, 325−326.
(23) Miller, D. G. *J. Phys. Chem.* **1958**, *62*, 767.
(24) Fujita, H.; Gosting, L. J. *J. Phys. Chem.* **1960**, *64*, 1256−1263.
(25) Hafskjold, B.; Ikeshoji, T. *Fluid Phase Equilib.* **1995**, *104*, 173−184.
(26) Hanot, V. P.; Platten, J. K.; Chavepeyer, G.; Larre, J. P. *Entropie* **1998**, *34* (214), 61−64.
(27) Havenga, E.; Leaist, D. G. *J. Chem. Soc., Faraday Trans.* **1998**, *94*, 3353−3358.

pressure and activity data to assess the approximation $\partial\mu_1/\partial C_2 \approx (D_{12}/D_{11})\ \partial\mu_1/\partial C_1$. That effort produced only qualitative agreement for each of the two values of $\partial\mu_1/\partial C_1$ obtained by differentiation of possibly inaccurate thermodynamic data.

The present work is also significant in that it provides direct access to chemical potential variations in ternary protein systems ranging from undersaturated to significantly supersaturated. Most equilibrium techniques are limited to salt−protein saturation conditions. On the isothermal protein−salt saturation curve, measurements are complicated by long equilibration times and subjective judgments of what constitutes equilibrium.

Most classical equilibrium techniques (isopiestic, vapor pressure, freezing-point depression) are insufficiently accurate to provide useful results, especially when the solute of interest is dilute. Electromotive force measurements require reversible solute-specific electrodes, which for proteins are typically unavailable and which could induce crystallization in supersaturated solution. Membrane osmometry is capable of dealing with dilute protein solutions, but lacks accuracy, and requires additional assumptions or generally unavailable data for complete determination of activity coefficients. Equilibrium dialysis and its variants also lack accuracy. Moreover, with either of the two latter techniques, the membrane poses precipitation problems for supersaturated solutions. Sedimentation equilibrium by ultracentrifugation requires long-duration experiments and is unsuitable for supersaturated solutions. Static light scattering, which can in principle be used for undersaturated and supersaturated systems obtains, at best, only the second virial coefficient, and has the disadvantage that additional assumptions or a generally unavailable thermodynamic derivative[28,29] ($\partial \ln \gamma_1/\partial m_2$ in our notation) is required to obtain the true concentration variation of the chemical potential.

In contrast, our technique requires only that the protein component be dilute with respect to the salt on a molar basis, and that binary thermodynamic data for the aqueous salt solution be available or measurable. Furthermore, our technique can be used to study supersaturated solutions as long as the experiment ends before the onset of precipitation.

Experimental Section

The materials, solution preparation procedures, apparatus, and density measurement procedures were described earlier.[5] Seikagaku six times recrystallized hen egg-white lysozyme chloride from one lot (E96301) was used to prepare solutions for all binary experiments reported here. Solutions used in binary experiment LC8 were prepared with lysozyme chloride from the lot E96301 bottle which, as described earlier,[5] appears to be slightly drier than lysozyme chloride from other bottles of the same lot. For the ternary experiments, the lot numbers used in each experiment were the same as for the corresponding NaCl concentrations of our earlier paper. The molecular masses of neutral lysozyme, NaCl, and H_2O were taken to be 14 307, 58.443, and 18.015 g mol^{-1},[5] respectively.

As before, all mutual diffusion coefficients were measured with the Gosting optical interferometric diffusiometer operating in its automated Rayleigh mode.[5,30] Separations of Creeth pairs were used to analyze fringe patterns as described elsewhere.[31,32] All runs had approximately 50 fringes. The procedures for measuring binary and ternary diffusion coefficients were described earlier.[5] For each pair of mean concentrations in the ternary case, two measurements were performed with α_1 near 0 and two with α_1 near 1, except at $C_2 = 1.30$ M (7.2 wt %) where we used $\alpha_1 = 0.8$ instead of 1.0 to avoid precipitation. Here, $\alpha_i \equiv R_i\Delta C_i/(R_1\Delta C_1 + R_2\Delta C_2)$ is the refractive index fraction due to the ith solute.[5]

Data analysis of the free-diffusion experiments[32] is based on the assumption that the concentration differences of the solutes across the initial boundary are small enough that the diffusion coefficients are constant and that Fick's second law

$$\frac{\partial C_i}{\partial t} = \sum_{j=1}^{n} D_{ij}\frac{\partial^2 C_j}{\partial x^2} \qquad 1 \le i \le n \tag{3}$$

can be applied, where n is the number of solutes.

Since our concentration differences across the initial free-diffusion boundary were small, the volume changes on mixing were also negligible. Thus, all the measured diffusion coefficients are given relative to the volume-fixed frame of reference defined by

$$\sum_{i=0}^{n} J_i\bar{V}_i = 0 \tag{4}$$

where J_i and $\bar{V}_i$ are the molar flux and partial molar volume of the ith component, respectively, and the subscript "0" denotes the solvent.

For pH 6.0, we again observed that lysozyme chloride precipitated from the supersaturated 1.30 M NaCl solutions, typically after 2 days.

Results

We report results for two series of binary experiments and one ternary series. In the first binary series, we measure the concentration dependence of the binary D_v for aqueous lysozyme chloride at pH 6.0 and compare it to the results at pH 4.5 reported earlier.[5] In the second binary series, we present the pH dependence of the binary D_v at 0.89 mM. The *ternary* diffusion coefficient measurements are used with eq 1 and the ORR to extract the derivatives of chemical potential with respect to composition at pH 4.5 and 6.0. They are also an important part of our program to develop a complete understanding of diffusive transport in the aqueous lysozyme chloride−NaCl system over the range of conditions relevant to crystal growth.

Binary D_v as a Function of Aqueous Lysozyme Chloride at pH 6.0. In this set of experiments, the mean lysozyme chloride concentration $\bar{C}_1$ ranged from 0.25 to 1.95 mM (3.6−27.9 mg/mL). Binary diffusion coefficients are shown in Figure 1, and are tabulated with the following supporting data in Table 1: $\bar{C}_1$, the mean lysozyme chloride concentration; ΔC_1, the lysozyme chloride concentration difference across the initial boundary; pH values for the bottom and top solutions; d_{bot} and d_{top}, the densities of the bottom and top solutions; Δt, a time offset for the inevitable deviation from exact step-function behavior of the solute distribution at the start of the clock; J, the total number of fringes; $\bar{V}_0$ and $\bar{V}_1$, the partial molar volumes of H_2O and lysozyme chloride at the mean concentration for each experiment, determined from the densities and concentrations of the reported solution pair;[5] $R_1 = J/\Delta C_1$, the refractive index increment with respect to J[33,34] at the mean concentration; and D_v, the mutual diffusion coefficient of lysozyme chloride in the volume-fixed reference frame. Because the HCl concentrations are much lower at pH 6.0 than at pH 4.5, there was no need to correct D_v for ternary behavior associated with the increased relative importance of HCl as the second solute at

(28) Scatchard, G. *J. Am. Chem. Soc*. **1946**, *68*, 2315−2319.
(29) Tanford, C. *Physical Chemistry of Macromolecules*; Wiley: New York, 1961.
(30) Gosting, L. J.; Kim, H.; Loewenstein, M. A.; Reinfelds, G.; Revzin, A. *Rev. Sci. Instrum*. **1973**, *44*, 1602−1609.
(31) Miller, D. G.; Albright, J. G. In *Measurement of the Transport Properties of Fluids*: *Experimental Thermodynamics*; Wakeham, W. A., Nagashima, A., Sengers, J. V., Eds.; Blackwell Scientific Publications: Oxford, 1991; pp 272−294.
(32) Miller, D. G.; Albright, J. G.; Mathew, R.; Lee, C. M.; Rard, J. A.; Eppstein, L. B. *J. Phys. Chem.* **1993**, *97*, 3885−3899.
(33) Miller, D. G.; Paduano, L.; Sartorio, R.; Albright, J. G. *J. Phys. Chem.* **1994**, *98*, 13,745−13,754.

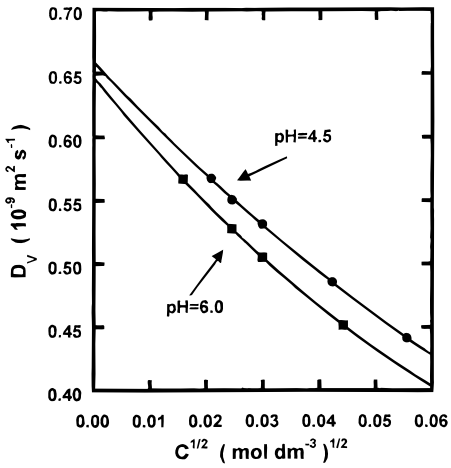

Figure 1. Diffusion coefficients of lysozyme chloride in H_2O at 25 °C versus lysozyme chloride concentration: ●, pH 4.5; ■, pH 6.0. Least-squares curves are described in the text.

Table 1. Binary Experimental and Derived Data at 25 °C, pH 6.0 (Series LC6−9)

expt	LC6	LC7	LC8	LC9
$\bar{C}_1$ (mM)	0.2497	0.6000	0.8900	1.9538
ΔC_1 (mM)	0.2863	0.3999	0.4118	0.6560
pH bottom	6.00	6.01	6.00	6.02
pH top	6.03	5.95	6.00	5.99
d_{bot} (g cm^{-3})	0.99869_6	1.00026_7	1.00157_1	1.00641_1
d_{top} (g cm^{-3})	0.99748_8	0.99869_3	0.99986_8	1.00373_2
Δt (s)	15	20	38	9
J_{measd}	37.096	51.042	52.482	83.458
$\bar{V}_1$ (10^3 cm^3 mol^{-1})	10.1_2	10.4_0	10.2_0	10.2_5
$\bar{V}_0$ (cm^3 mol^{-1})	18.06_9	18.06_7	18.06_8	18.06_8
R_1 (10^5 dm^3 mol^{-1})	1.296	1.276	1.274	1.272
D_v (measd) (10^{-9} m^2 s^{-1})	0.5670	0.5280	0.5054	0.4518

low lysozyme chloride concentrations.[5] The binary D_v values reported are internally consistent within the series to better than 0.1%. The accuracy is significantly better than 1%, but ultimately depends on lysozyme purity.

Binary D_v at 0.89 mM Lysozyme Chloride as a Function of pH. Binary diffusion coefficients for a series of experiments performed at a mean lysozyme chloride concentration of 0.89 mM in the pH range 4.0−6.0 are shown in Figure 2, and tabulated with supporting data in Table 2.

The diagnostic Ω values[5,35] are small, as previously observed for solutions prepared from the same lysozyme chloride lots. This reconfirms that the diffusion is essentially binary, that the concentration dependence of the diffusion coefficient is unimportant, and that the lysozyme was of good purity.[5]

Ternary $(D_{ij})_v$ at pH 6.0 and a Lysozyme Chloride Concentration of 0.60 mM. Four ternary experiments were performed at each of five different mean compositions. Each experiment in a set of four had the same mean NaCl and 0.6 mM lysozyme chloride concentrations, but different concentration differences of the solutes across the diffusion boundary. The mean lysozyme chloride concentration and the five mean

(34) The conventional refractive index increment, with respect to the fringe number J, is R_i. The more fundamental quantity, $R_i^* = (a/\lambda)R_i$, can be computed from the tabulated quantities, where λ is the optical wavelength and a is the internal cell dimension along the optical axis, which in our case are 543.5 nm and 2.5074 cm, respectively. Since extraction of diffusion coefficients depends only on the ratio $R_1/R_2 = R_1^*/R_2^*$, either increment can be used. The refractive index increment with respect to J, which might also be called the "fringe number increment", has the advantage that the cell size a is not required.

(35) Creeth, J. M.; Gosting, L. J. *J. Phys. Chem.* **1958**, *62*, 58−65.

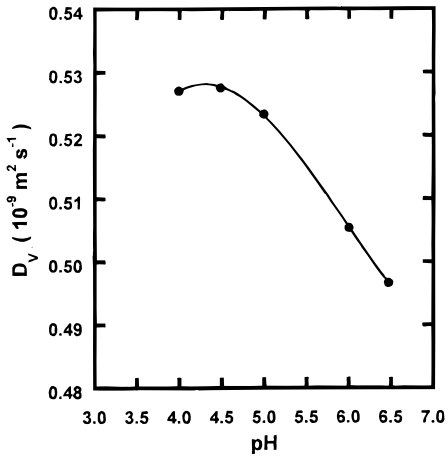

Figure 2. Diffusion coefficients of 0.89 mM lysozyme chloride in H_2O at 25 °C versus pH. The fitted curve is piecewise quadratic.

Table 2. Binary Experimental and Derived Data at 25 °C, $\bar{C}_1$ = 0.89 M (Series LC10−12)

expt	LC10	LC2[a]	LC11	LC8	LC12
$\bar{C}_1$ (mM)	0.8914	0.8914	0.8914	0.8900	0.8913
ΔC_1 (mM)	0.3364	0.3364	0.3364	0.4118	0.3363
pH bottom	3.98	4.48	4.99	6.00	6.48
pH top	4.01	4.48	4.99	6.00	6.46
d_{bot} (g cm^{-3})	1.00150_4	1.00149_4	1.00149_4	1.00157_1	1.00143_8
d_{top} (g cm^{-3})	1.00008_6	1.00008_1	1.00007_7	0.99986_8	1.00006_6
Δt (s)	10	9	8	38	18
J_{measd}	43.742	43.639	43.663	52.482	43.473
$\bar{V}_1$ (10^3 cm^3 mol^{-1})	10.1_3	10.1_4	10.1_2	10.2_0	10.2_6
$\bar{V}_0$ (cm^3 mol^{-1})	18.06_8	18.06_9	18.06_9	18.06_8	18.06_7
R_1 (10^5 dm^3 mol^{-1})	1.300	1.297	1.298	1.274	1.293
D_v (measd) (10^{-9} m^2 s^{-1})	0.5271	0.5276	0.5234	0.5054	0.4967
pH av	3.99	4.48	4.99	6.00	6.47

[a] Data from this experiment were reported in ref 5.

Table 3. Ternary Experimental Data at 25 °C, [NaCl] = 0.25 M (Series LNC6)

expt	LNC61	LNC62	LNC63	LNC64
$\bar{C}_1$ (mM)	0.6000	0.6000	0.6000	0.6000
$\bar{C}_2$ (M)	0.2500	0.2500	0.2500	0.2500
ΔC_1 (mM)	0.4000	0.0000	0.4000	0.0000
ΔC_2 (M)	0.0000	0.1108	0.0000	0.1108
pH bottom	5.99	6.02	6.00	6.02
pH top	6.00	6.01	6.00	5.97
d_{bot} (g cm^{-3})	1.01061_2	1.01202_3	1.01061_9	1.01202_5
d_{top} (g cm^{-3})	1.00896_1	1.00753_5	1.00895_5	1.00753_9
Δt (s)	37	9	21	8
J_{measd}	51.775	50.823	51.767	50.821
J_{calcd}	51.775	50.817	51.767	50.827
D_A (measd) (10^{-9} m^2 s^{-1})	0.1237	1.662	0.1236	1.651
D_A (calcd) (10^{-9} m^2 s^{-1})	0.1237	1.724	0.1237	1.724

NaCl concentrations, 0.25, 0.50, 0.65, 0.90, and 1.30 M (1.4, 2.8, 3.7, 5.1, and 7.2 wt %), were identical to those previously investigated at pH 4.5.[5]

Data from each experiment are presented in Tables 3−7. Besides the symbols introduced in Table 1, the data shown are the following: $\bar{C}_2$, the mean NaCl concentration; ΔC_2, the NaCl concentration difference across the free-diffusion starting boundary; J_{measd}, the total number of fringes observed; J_{calcd}, obtained from the ΔC_i and "refractive index increments with respect to J" R_i defined below; and D_A, the reduced-height-area ratio,[36] obtained as described in ref 5.

(36) Dunlop, P. J.; Gosting, L. J. *J. Am. Chem. Soc.* **1955**, *77*, 5238−5249.

Table 4. Ternary Experimental Data at 25 °C, [NaCl] = 0.50 M (Series LNC8)

expt	LNC81	LNC82	LNC83	LNC84
$\bar{C}_1$ (mM)	0.6000	0.6000	0.6000	0.6000
$\bar{C}_2$ (M)	0.5000	0.5000	0.5000	0.5000
ΔC_1 (mM)	0.4000	0.0000	0.4000	0.0000
ΔC_2 (M)	0.0000	0.1136	0.0000	0.1136
pH bottom	6.01	6.01	6.01	6.01
pH top	6.01	6.00	6.01	6.01
d_{bot} (g cm^{-3})	1.02061_9	1.02205_1	1.02061_7	1.02205_0
d_{top} (g cm^{-3})	1.01897_9	1.01751_8	1.01897_5	1.01751_8
Δt (s)	30	8	16	7
J_{measd}	51.148	50.910	51.137	51.006
J_{calcd}	51.129	50.964	51.157	50.951
D_A (measd) (10^{-9} m^2 s^{-1})	0.1186	1.594	0.1187	1.587
D_A (calcd) (10^{-9} m^2 s^{-1})	0.1186	1.632	0.1187	1.632

Table 5. Ternary Experimental Data at 25 °C, [NaCl] = 0.65 M (Series LNC9)

expt	LNC91	LNC93	LNC94	LNC96c
$\bar{C}_1$ (mM)	0.6000	0.6000	0.6000	0.6000
$\bar{C}_2$ (M)	0.6500	0.6500	0.6500	0.6500
ΔC_1 (mM)	0.4000	0.4000	0.0000	0.0000
ΔC_2 (M)	0.0000	0.0000	0.1108	0.1108
pH bottom	6.02	6.00	6.01	6.00
pH top	5.99	6.00	6.00	5.99
d_{bot} (g cm^{-3})	1.02657_2	1.02657_3	1.02794_7	1.02794_4
d_{top} (g cm^{-3})	1.02492_9	1.02493_5	1.02355_9	1.02355_5
Δt (s)	24	8	18	5
J_{measd}	51.094	51.148	49.064	49.183
J_{calcd}	51.128	51.114	49.129	49.118
D_A (meas) (10^{-9} m^2 s^{-1})	0.1166	0.1167	1.578	1.581
D_A (calc) (10^{-9} m^2 s^{-1})	0.1167	0.1167	1.617	1.618

Table 6. Ternary Experimental Data at 25 °C, [NaCl] = 0.90 M (Series LNC7)

expt	LNC71	LNC72b	LNC73	LNC74b
$\bar{C}_1$ (mM)	0.6000	0.6000	0.6000	0.6000
$\bar{C}_2$ (M)	0.9000	0.9000	0.8999	0.8999
ΔC_1 (mM)	0.4000	0.0000	0.4000	0.0001
ΔC_2 (M)	0.0000	0.1108	0.0000	0.1109
pH bottom	6.00	6.02	6.02	6.01
pH top	6.01	6.02	6.00	6.00
d_{bot} (g cm^{-3})	1.03644_4	1.03776_5	1.03643_2	1.03776_6
d_{top} (g cm^{-3})	1.03478_2	1.03344_3	1.03473_6	1.03335_5
Δt (s)	15	9	20	10
J_{measd}	51.707	48.408	51.522	48.367
J_{calcd}	51.614	48.369	51.615	48.407
D_A (meas) (10^{-9} m^2 s^{-1})	0.1136	1.577	0.1133	1.575
D_A (calc) (10^{-9} m^2 s^{-1})	0.1135	1.615	0.1135	1.613

Table 8 shows data derived from these five sets of experiments, including $\bar{\bar{C}}_i$, the average concentration of solute i in all eight solutions (four bottom solutions and four top solutions) for each set-mean composition; $\bar{d}$ and $H_i \approx \partial d/\partial C_i$ (see eq 3 in ref 5), obtained as functions of C_1 and C_2 by a linear regression of the density data from all solution pairs shown in Tables 3−7, as well as solutions used in a few unsuccessful diffusion experiments; the partial molar volumes $\bar{V}_1$ and $\bar{V}_2$ of the solutes (calculated using eq A-7 of ref 13) at the mean concentration in each set of experiments; $\bar{V}_0$, the partial molar volume of H_2O,[37] implicit in

$$\sum_{i=0}^{n} C_i \bar{V}_i = 1 \qquad (5)$$

(37) Rard, J. A.; Albright, J. G.; Miller, D. G.; Zeidler, M. E. *J. Chem. Soc., Faraday Trans.* **1996**, *92*, 4187−4197.

Table 7. Ternary Experimental Data at 25 °C, [NaCl] = 1.30 M (Series LNC10)

expt	LNC101e	LNC102	LNC103	LNC104
$\bar{C}_1$ (mM)	0.5999_5	0.5999_5	0.5999_5	0.5999_5
$\bar{C}_2$ (M)	1.2999	1.2999	1.2999	1.2999
ΔC_1 (mM)	0.3199_5	0.0000	0.3199_5	0.0000
ΔC_2 (M)	0.02212	0.11083	0.02212	0.11084
pH bottom	6.01	6.00	6.00	6.01
pH top	6.01	6.01	5.99	6.00
d_{bot} (g cm^{-3})	1.05216_2	1.05321_8	1.05216_2	1.05324_1
d_{top} (g cm^{-3})	1.04999_8	1.04892_6	1.05001_3	1.04894_8
Δt (s)	132	8	18	5
J_{measd}	50.077	47.146	50.180	47.222
J_{calcd}	50.129	47.181	50.128	47.187
D_A (measd) (10^{-9} m^2 s^{-1})	0.1474	1.588	0.1473	1.584
D_A (calcd) (10^{-9} m^2 s^{-1})	0.1481	1.626	0.1481	1.626

and $R_i = \partial J/\partial C_i$, the refractive index increments with respect to J,[33,34] obtained to good approximation by linear regression of the J values versus ΔC_i for all four experiments of each set, and used to determine J_{calc}. Also included are the ratio S_A/I_A,[32,38] a diagnostic which if smaller than about 0.2 implies strong sensitivity of the diffusion coefficients to errors in the computations by which they are extracted from the fringe data; the eigenvalues of the diffusion coefficient matrix, λ_1 and λ_2 ($\lambda_1 < \lambda_2$), associated with lysozyme chloride and NaCl, respectively; and $(D_{ij})_v$, the measured diffusion coefficients in the volume-fixed frame. The errors shown for $(D_{ij})_v$ are approximately *4 times* the standard error of the coefficients as determined from the propagation-of-error equations using the full covariance matrix of the least-squares parameters.[32,33,39]

Equations to relate the volume-fixed frame to the solvent-fixed frame[10,13,14] require the $\bar{V}_i$ of all components. Parts A and B of Table 9 give the values of $(D_{ij})_0$ in the solvent-fixed frame (defined by $J_0 = 0$)[10,40] for pH 4.5 and 6.0, respectively, with the pH 4.5 values calculated from the volume-fixed $(D_{ij})_v$ reported earlier.[5]

Discussion of Diffusion Coefficients

Binary D_v of Aqueous Lysozyme Chloride. Figure 1 shows plots of D_v versus $\sqrt{C_1}$ for pH 4.5 data previously reported[5] and for pH 6.0 data reported in Table 1. The plots are nearly linear. The equation $D_v = 0.6470(1 - 8.38\sqrt{C_1} + 35.1C_1)$ was fitted to the pH 6.0 data, where D_v and C_1 have units of 10^{-9} m^2 s^{-1} and M, respectively.

Extrapolating to zero protein concentration, we find that D_v lies about 2% below the infinite-dilution value at pH 4.5.[5] Both values exceed, by about 10%, the value extrapolated to zero protein concentration by Cadman, Fleming, and Guy.[41] Those authors performed Goüy interferometric measurements at 25 °C over a range of lysozyme chloride concentrations, and fitted a linear relation to the measured diffusion coefficients as a function of the mass fraction of lysozyme chloride in solution. The solutions were in the pH range 3.3−3.6, and no correction was made for the presence of HCl.[5]

As in our earlier work,[5] we can compute the charge of the lysozyme cation. We take the limiting tracer diffusion coefficient of the aqueous lysozyme cation to be 0.12×10^{-9}m^2 s^{-1}, on the basis of the data shown in Figure 3 of ref 5. At pH 6.0, this gives a value of $z_P = 6._{45}$ for the charge of the lysozyme cation.

(38) Fujita, H.; Gosting, L. J. *J. Am. Chem. Soc.* **1956**, *78*, 1099−1106.
(39) Miller, D. G.; Sartorio, R.; Paduano, L.; Rard, J. A.; Albright, J. G. *J. Solution Chem.* **1996**, *25*, 1185−1211.
(40) Kirkwood, J. G.; Baldwin, R. L.; Dunlop, P. J.; Gosting, L. J.; Kegeles, G. *J. Chem. Phys.* **1960**, *33*, 1505−1513.
(41) Cadman, A. D.; Fleming, R.; Guy, R. H. *Biophys. J.* **1981**, *37*, 569−574.

Table 8. Derived Ternary Diffusion Data at 25 °C, pH = 6.0

series	LNC6	LNC8	LNC9	LNC7	LNC10
$\bar{C}_1$ (mM)	0.6000	0.6000	0.6000	0.6000	0.5999_5
$\bar{C}_2$ (M)	0.2500	0.5000	0.6500	0.9000	1.2999
$\bar{d}$ (g cm^{-3})	1.00978_3	1.01979_1	1.02575_3	1.03560_7	1.05108_6
H_1 (10^3 g mol^{-1})	4.14_4	4.10_2	4.10_3	4.14_4	4.06_2
H_2 (10^3 g mol^{-1})	0.040_5	0.039_9	0.039_6	0.039_2	0.038_7
$\bar{V}_1$ (cm^3 mol^{-1})	10192	10232	10229	10184	10262
$\bar{V}_2$ (cm^3 mol^{-1})	18.014	18.602	18.879	19.324	19.746
$\bar{V}_0$ (cm^3 mol^{-1})	18.066	18.062	18.059	18.053	18.046
R_1 (10^2 dm^3 mol^{-1})	1294	1278	1278	1290	1272
R_2 (10^2 dm^3 mol^{-1})	4.585	4.485	4.432	4.365	4.257
S_A/I_A	2.734	2.773	2.709	2.723	2.849
λ_1 (10^{-9} m^2 s^{-1})	0.1194	0.1057	0.1130	0.1103	0.0996
λ_2 (10^{-9} m^2 s^{-1})	1.461	1.462	1.456	1.457	1.475
$(D_{11})_v$ (10^{-9} m^2 s^{-1})	0.1204 ± 0.0001	0.1140 ± 0.0001	0.1113 ± 0.0001	0.1069 ± 0.0001	0.1011 ± 0.0001
$(D_{12})_v$ (10^{-9} m^2 s^{-1})	0.000155 ± 0.000002	0.000104 ± 0.000002	0.000094 ± 0.000001	0.000086 ± 0.000002	0.000082 ± 0.000002
$(D_{21})_v$ (10^{-9} m^2 s^{-1})	9.0 ± 0.2	12.4 ± 0.2	14.7 ± 0.1	18.7 ± 0.3	26.0 ± 0.2
$(D_{22})_v$ (10^{-9} m^2 s^{-1})	1.460 ± 0.001	1.455 ± 0.001	1.456 ± 0.001	1.461 ± 0.001	1.474 ± 0.001

Table 9

A. Solvent-Fixed Ternary Diffusion Coefficients for pH 4.5, $C_1 = 0.6$ mM

	$C_2 = 0.25$ M	$C_2 = 0.50$ M	$C_2 = 0.65$ M	$C_2 = 0.90$ M	$C_2 = 1.30$ M
$(D_{11})_0$ (10^{-9} m^2 s^{-1})	0.1263	0.1191	0.1156	0.1111	0.1041
$(D_{12})_0$ (10^{-9} m^2 s^{-1})	0.000186	0.000124	0.000112	0.000104	0.000097
$(D_{21})_0$ (10^{-9} m^2 s^{-1})	10.3	14.5	17.0	21.2	28.9
$(D_{22})_0$ (10^{-9} m^2 s^{-1})	1.466	1.469	1.474	1.488	1.516

B. Solvent-Fixed Ternary Diffusion Coefficients for pH 6.0, $C_1 = 0.6$ mM

	$C_2 = 0.25$ M	$C_2 = 0.50$ M	$C_2 = 0.65$ M	$C_2 = 0.90$ M	$C_2 = 1.30$ M
$(D_{11})_0$ (10^{-9} m^2 s^{-1})	0.1212	0.1149	0.1122	0.1078	0.1021
$(D_{12})_0$ (10^{-9} m^2 s^{-1})	0.000172	0.000121	0.000111	0.000104	0.000101
$(D_{21})_0$ (10^{-9} m^2 s^{-1})	9.4	13.1	15.6	20.0	28.1
$(D_{22})_0$ (10^{-9} m^2 s^{-1})	1.467	1.469	1.475	1.488	1.514

With this charge, the binary-system Nernst−Hartley equation gives the limiting D_v. The chloride ion diffusion coefficient was taken to be 2.03×10^{-9}m^2 s^{-1}.[42]

On the other hand, we can use a Harned-type analysis[1] of the concentration dependence of the diffusion coefficients, where in our application the protein charge z_P is adjusted to match the diffusion data. This assumes that the slope of D_v versus $\sqrt{C_1}$ is due to the concentration dependence of the activity coefficient, and that the concentration dependence of the diffusion coefficient is given by the Debye−Hückel limiting law for dilute solutions. The resulting charge of the protein cation is $z_P = 4.3$. However, this value should be interpreted with care, since the charge is high, and the lysozyme cation is not spherical with a central charge, as assumed for the Debye−Hückel limiting law.

Comparison of z_P values computed for pH 4.5 ($z_P = 6.7$ and 3.9 from the limiting diffusion coefficient and the Debye−Hückel limiting law, respectively)[5] to those at pH 6.0 shows the charge at pH 6.0 to be less, as expected. The slope of D_v versus $\sqrt{C_1}$ is only slightly greater at pH 6.0 than at pH 4.5, implying a marginally greater effective charge. Again, interpretation is complicated by the high charge and asphericity of the lysozyme cation.

Figure 2 shows D_v versus pH for 0.89 mM lysozyme chloride solutions. Interpretation of these data, which exhibit an apparent maximum at low pH, must consider several factors. These include the actual charge, the consequent dragging of lysozyme cations by more mobile chloride anions, and the concentration dependence of the lysozyme chloride activity coefficient as a function of pH. As the pH increases toward lysozyme's isoelectric value of 11,[43] the protein charge decreases. Along with a corresponding decrease in the number of associated chloride anions and the cations of lysozyme they drag, this contributes to the expected decrease in D_v. At the lowest pH values, emergence of HCl as a third component and of hydrogen ions as charge carriers will reduce the flux of lysozyme cations dragged by chloride anions. Since the available data do not permit a definitive assignment of the pH contribution to the variation in D_v, we have made no attempt to correct for the pH-dependent contribution of HCl.

Ternary $(D_{ij})_v$ at pH 6.0 and Lysozyme Chloride Concentration of 0.60 mM. The dependence of the ternary diffusion coefficients D_{ij} on NaCl concentration at pH 4.5 and 6.0 is shown in Figure 3 for $0.25 \text{ M} \leq C_2 \leq 1.30$ M.

The values of D_{11} (Figure 3a), corresponding to diffusion of lysozyme chloride due to its own gradient, decrease with increasing NaCl concentration at both pH values. The increase of aqueous NaCl viscosity with increasing C_2 over the same C_2 range[44] accounts for most, but not all, of the decrease in D_{11}. At a given NaCl concentration, the D_{11} values are smaller at the higher pH. Because the high NaCl concentration greatly reduces the dragging of lysozyme cations by chloride anions, the residual effects of those chlorides associated with lysozyme should be lower at the higher pH. Other effects might also be important, including differences in the degree of hydration of the protein as a function of pH.

The values of D_{12} (Figure 3b) are very small and, when expressed in terms of thermodynamic transport coefficients (D_{12}

(42) Mills, R. *J. Phys. Chem.* **1957**, *61*, 1631−1634.

(43) Tanford, C.; Wagner, M. L. *J. Am. Chem. Soc.* **1954**, *76*, 3331−3336.

(44) Jones, G.; Christian, S. M. *J. Am. Chem. Soc.* **1937**, *59*, 484−486.

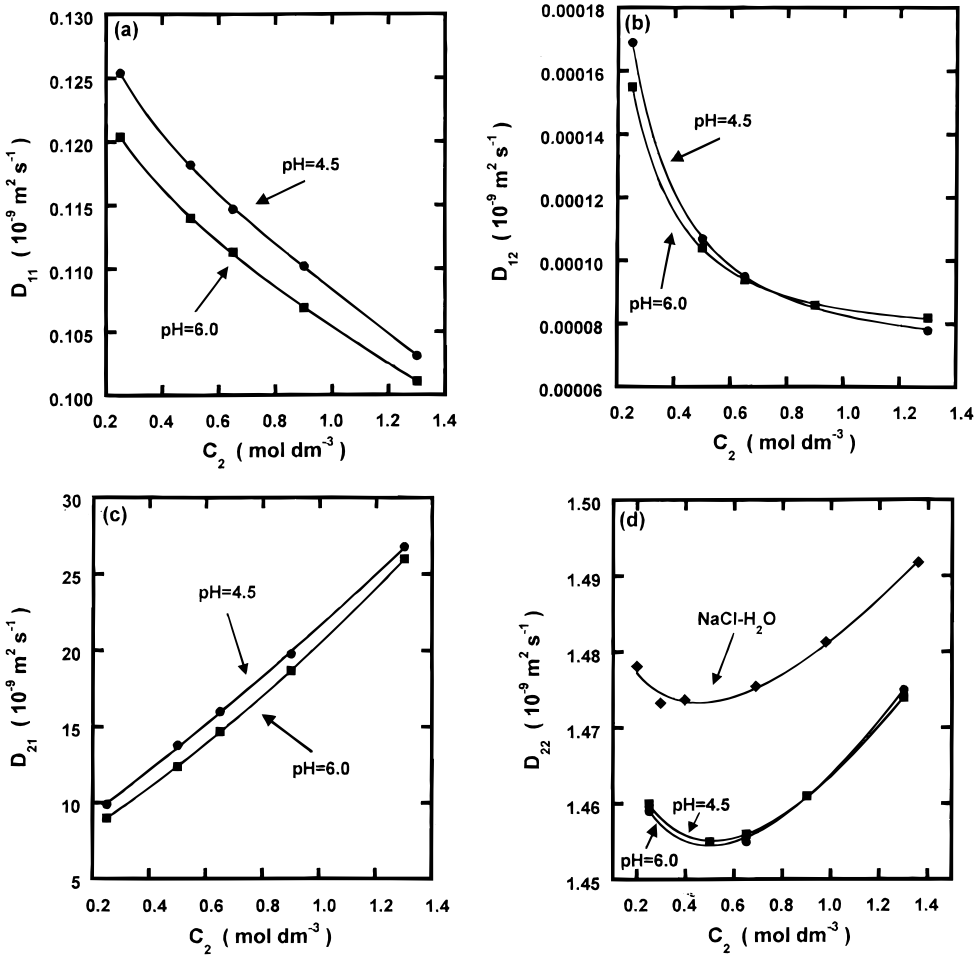

Figure 3. Elements of the matrix of diffusion coefficients for the ternary system lysozyme chloride + NaCl + H_2O at pH 6.0 and 25 °C: ●, pH 4.5; ■, pH 6.0. (a, top left) D_{11}, least-squares curves are cubic in $C_2^{1/2}$; (b, top right) D_{12}, least-squares curves are of the form $a_0C_2^{-1/2} + a_1 + a_2C_2^{1/2}$; (c, bottom left) D_{21}, least-squares curves are quadratic in C_2; (d, bottom right) D_{22} and D_v for binary diffusion of NaCl in H_2O (shown by ◆), least-squares curves are cubic in $C_2^{1/2}$.

$= L_{11}\mu_{12} + L_{12}\mu_{22}$), result from near-cancellation of two terms with opposite signs and similar magnitudes. The D_{12} values at lower concentration for pH 6.0 are larger than those at pH 4.5, but cross over around $C_2 = 0.6$ M. The data are believed to be precise enough that this effect is probably real.

Values of D_{21} (Figure 3c) are large and increase rapidly with increasing C_2 for both pH values, but at each NaCl concentration are slightly lower at pH 6.0. The difference between D_{21} values at pH 4.5 and 6.0 remains nearly constant as C_2 varies, as discussed below in terms of the decreased charge of the lysozyme cation as the isoelectric pH is approached. The ratio D_{21}/D_{11} indicates that each mole of lysozyme chloride cotransports 257 ± 2 mol of NaCl in a uniform 1.30 M NaCl solution at pH 6.0. This is identical, within experimental error, to the value of 260 ± 2 at pH 4.5.[5] The ratio D_{21}/D_{11} increases sharply and nearly linearly with NaCl concentration. The values at pH 6.0 exceed those at pH 4.5 by 1−8%. The dependence of D_{21} on NaCl concentration can be interpreted in terms of excluded volume or "salting-out" effects. The relative merits of those interpretations, in light of precision ternary diffusion data to be published for lysozyme chloride in aqueous solutions of sodium, potassium, magnesium, calcium, and ammonium chlorides, will be thoroughly explored elsewhere.

The values of D_{22} (Figure 3d) are nearly the same for both pH values. Since this is the coefficient for NaCl diffusion due to its own gradient, no pH dependence is expected.

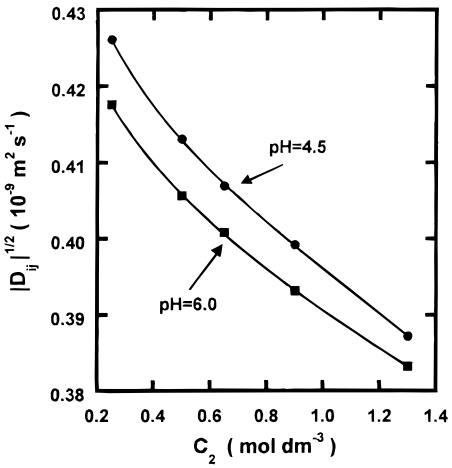

Figure 4. Square root of the determinant of the diffusion coefficient matrix for the ternary system lysozyme chloride + NaCl + H_2O at pH 6.0 and 25 °C: ●, pH 4.5; ■, pH 6.0. Least-squares curves are cubic in $C_2^{1/2}$.

Examination of the Determinant. Figure 4 shows the square root of the diffusion coefficient matrix, $|\mathbf{D}| \equiv (D_{11})_v(D_{22})_v - (D_{12})_v(D_{21})_v$, as a function of C_2 for both pH values. At each NaCl concentration, the pH 6.0 values of $|\mathbf{D}|$ are consistently lower than those for pH 4.5. For each pH, $|\mathbf{D}|$ decreases by about 10% as C_2 increases from 0.25 M into the supersaturated

region at 1.30 M. The plots suggest that if the determinant vanishes for 0.60 mM lysozyme chloride, it does so well above 1.30 M. Consequently, the data indicate that if the spinodal curve intersects the $C_1 = 0.6$ mM line in the $C_1 - C_2$ plane, it does so at an NaCl concentration well in excess of 1.30 M.[5]

Partial Molar Volumes. The partial molar volume $\bar{V}_1$ of lysozyme chloride is independent of C_2 within experimental error, and apparently does not depend significantly on pH either, although experimental error may hide a small effect.

In contrast, the partial molar volume $\bar{V}_2$ of NaCl increases with increasing C_2. This behavior is very similar to that in binary NaCl + H_2O. However, the values in the ternary system are slightly higher, consistent with an excluded volume effect. Again, there does not seem to be a significant pH dependence.

Other Measured or Calculated Quantities. The parameters H_1 and H_2, defined in eq 3 of ref 5 and corresponding approximately to the derivatives of solution density with respect to C_1 and C_2, respectively, are independent of C_2 and pH within experimental error.

The R_1 and R_2 values relating J to ΔC_1 and ΔC_2 are nearly the same for both pH values at a given mean concentration. Within experimental error, R_1 is apparently independent of concentration, whereas R_2 decreases slowly with increasing C_2, as it also does in binary aqueous NaCl.

The eigenvalue λ_1 (associated with lysozyme chloride) at pH 4.5 is approximately 3% higher than at pH 6.0 at each concentration, and decreases with increasing NaCl concentration at both pH values. At pH 4.5 and 6.0, the λ_2 values (associated with NaCl) are approximately equal, as expected, and nearly independent of C_2. At pH 6.0, the values of λ_2 agree even more closely with measured values of D_v in aqueous NaCl[46] than at pH 4.5, being within 1% of and always lower than D_v.

Use of Irreversible Thermodynamics and Diffusion Data To Calculate Chemical Potential Derivatives

Our lysozyme chloride−NaCl−H_2O system has an interesting and useful attribute characteristic of many biological systems: the molar concentrations of a macromolecule and the supporting electrolyte are small and large, respectively. In this special case, it is possible to estimate the chemical potential derivatives μ_{11} and μ_{22}, as will become clear below.

The molality cross-derivative relation, eq 1, comes from classical thermodynamics. From eq 1, an expression can be derived relating the four molarity partial derivatives μ_{ij}.[11] A second equation, the ternary ORR of irreversible thermodynamics, relates the four $(D_{ij})_0$ values and the four μ_{ij} values.[13,14] Therefore, we can get the two off-diagonal derivatives μ_{ij} ($i \neq j$) from these two relations, provided that suitably accurate approximations are available for the self-derivatives of the lysozyme chloride and sodium chloride chemical potentials, μ_{11} and μ_{22}, respectively.

The ORR are of considerable theoretical interest, and for this reason have been rigorously tested.[11,13−22] However, particularly for bulk diffusion, they have had little practical application.

Now, in an important application, we demonstrate use of the ORR to extract from our data thermodynamic properties that would otherwise be inaccessible. From binary thermodynamic data for the more concentrated solute and diffusion coefficients (transport data), it is possible to use the ORR to get the thermodynamic derivatives μ_{ij} even for supersaturated solutions. We will illustrate this result for the system lysozyme chloride−NaCl−H_2O, since it fulfills the required conditions. In addition, we can calculate the protein charge by least-squares fitting to an appropriate functional form, the thermodynamic derivative, μ_{12}, over the range of NaCl concentrations at constant lysozyme concentration.

We now review the thermodynamic equations which lead to our results.

Fundamental Equations. Our analysis is in terms of quantities referred to a solvent-fixed reference frame, identified by a subscript 0, with the "diffusion Onsager coefficients" denoted by $(L_{ij})_0$. As noted in the Results, the solvent-fixed $(D_{ij})_0$ values shown in Table 8 are obtained from experimental volume-fixed $(D_{ij})_v$ by standard equations involving the $\bar{V}_i$.[10,13,14]

It has been shown[10,13,14] that the $(L_{ij})_0$ and $(D_{ij})_0$ are related (in matrix form) by

$$\begin{bmatrix}(D_{11})_0 & (D_{12})_0\\ (D_{21})_0 & (D_{22})_0\end{bmatrix} = \begin{bmatrix}(L_{11})_0\mu_{11} + (L_{12})_0\mu_{21} & (L_{11})_0\mu_{12} + (L_{12})_0\mu_{22}\\ (L_{21})_0\mu_{11} + (L_{22})_0\mu_{21} & (L_{21})_0\mu_{12} + (L_{22})_0\mu_{22}\end{bmatrix} \quad (6)$$

The inverse relation is

$$\begin{bmatrix}(L_{11})_0 & (L_{12})_0\\ (L_{21})_0 & (L_{22})_0\end{bmatrix} = \frac{1}{\mu_{11}\mu_{22} - \mu_{12}\mu_{21}} \times \begin{bmatrix}\mu_{22}(D_{11})_0 - \mu_{21}(D_{12})_0 & \mu_{11}(D_{12})_0 - \mu_{12}(D_{11})_0\\ \mu_{22}(D_{21})_0 - \mu_{21}(D_{22})_0 & \mu_{11}(D_{22})_0 - \mu_{12}(D_{21})_0\end{bmatrix} \quad (7)$$

Since the ORR, $(L_{12})_0 = (L_{21})_0$,[13,14] apply to the solvent-fixed frame, eq 7 yields eq 2.

From the cross-derivative expression eq 1 and the relations between C_i and m_i, we can show that[11]

$$\frac{1}{C_0M_0}\frac{\partial\mu_1}{\partial m_2} = \mu_{12}(1 - C_2\bar{V}_2) - \mu_{11}C_1\bar{V}_2 = \mu_{21}(1 - C_1\bar{V}_1) - \mu_{22}C_2\bar{V}_1 = \frac{1}{C_0M_0}\frac{\partial\mu_2}{\partial m_1} \quad (8)$$

where M_0 is the molecular mass of H_2O.

The general thermodynamic expressions for μ_{ij} in terms of volume concentrations and the corresponding mean ionic activity coefficients y_i for volume concentrations have been derived.[11] Let z_P, z_{Na}, and z_{Cl} be the absolute values of the charges on the protein cation, sodium cation, and chloride ion common to the salt and protein, respectively. Furthermore, Na^+ and Cl^- are univalent, i.e., $z_{Na} = z_{Cl} = 1$. We have also assumed that lysozyme chloride has stoichiometry $LyCl_{z_p}$. Consequently, the cation stoichiometric coefficients[47] r_{1c} of $LyCl_{z_p}$ and r_{2c} of NaCl are both unity. With these conditions, the μ_{ij} for our particular case can be written in matrix form as

$$\begin{bmatrix}\mu_{11} & \mu_{12}\\ \mu_{21} & \mu_{22}\end{bmatrix} = RT \times \begin{bmatrix}\frac{1}{C_1} + \frac{z_P^2}{z_PC_1 + C_2} + (z_P + 1)\frac{\partial \ln y_1}{\partial C_1} & \frac{z_P}{z_PC_1 + C_2} + (z_P + 1)\frac{\partial \ln y_1}{\partial C_2}\\ \frac{z_P}{z_PC_1 + C_2} + 2\frac{\partial \ln y_2}{\partial C_1} & \frac{1}{C_2} + \frac{1}{z_PC_1 + C_2} + 2\frac{\partial \ln y_2}{\partial C_2}\end{bmatrix} \quad (9)$$

(45) Vitagliano, V.; Sartorio, R. *J. Phys. Chem.* **1970**, *74*, 2949−2956.
(46) Rard, J. A.; Miller, D. G. *J. Solution Chem.* **1979**, *8*, 701−716.
(47) Miller, D. G. *J. Phys. Chem.* **1967**, *71*, 616−632.

Table 10

A. Chemical Potentials and Derivatives for pH 4.5, $C_1 = 0.6$ mM

	$C_2 = 0.25$ M	$C_2 = 0.50$ M	$C_2 = 0.65$ M	$C_2 = 0.90$ M	$C_2 = 1.30$ M
μ_{11}/RT (M^{-1})	1984	1827	1790	1756	1729
μ_{22}/RT (M^{-1})	7.267	3.732	2.924	2.183	1.600
μ_{12}/RT (M^{-1})	35.0	19.2	15.5	11.8	10.1
μ_{21}/RT (M^{-1})	53.7	38.4	34.7	31.9	31.1
$(\mu_1 - \mu_1^*)/RT$	−12.21	−5.89	−3.36	0.00	4.36

B. Chemical Potentials and Derivatives for pH 6.0, $C_1 = 0.6$ mM

	$C_2 = 0.25$ M	$C_2 = 0.50$ M	$C_2 = 0.65$ M	$C_2 = 0.90$ M	$C_2 = 1.30$ M
μ_{11}/RT (M^{-1})	1918	1793	1764	1737	1716
μ_{22}/RT (M^{-1})	7.275	3.733	2.923	2.181	1.596
μ_{12}/RT (M^{-1})	29.9	15.2	12.3	10.0	8.9
μ_{21}/RT (M^{-1})	48.6	34.3	31.8	30.0	30.1
$(\mu_1 - \mu_1^*)/RT$	−10.00	−4.79	−2.74	0.00	3.69

Note that the quantity $z_P C_1 + C_2$ is equivalent to the total normality N of our ternary solution.

Evaluation of the μ_{ij}. With the above equations, we now show how to compute the partial derivatives of the chemical potentials, μ_{ij}, for the case in which the molarity of at least one component is very low, a common situation in multicomponent protein solutions.

(i) Calculation of μ_{11}. From eq 9, we can rewrite μ_{11} as

$$\mu_{11} = \frac{RT}{C_1}\left[1 + \frac{z_P^2 C_1}{z_P C_1 + C_2} + (z_P + 1)C_1\frac{\partial \ln y_1}{\partial C_1}\right] \quad (10)$$

from which we can see that the first term is dominant for small C_1. Values of μ_{11} calculated by retaining the first two terms are given in Table 10 for pH 4.5 and 6.0 for all NaCl concentrations.

Consideration of light-scattering measurements of the second virial coefficient of lysozyme chloride by Guo et al.[48] and formulas that relate μ_{11} to the second virial coefficient[28] suggests that the absolute error associated with dropping the third term in eq 10 does not exceed 10%. For the application below, the accuracy required of μ_{11} need not be high, so that the values calculated from eq 10 are satisfactory.

(ii) Calculation of μ_{22}. Since $C_2 \gg C_1$, we observe, not surprisingly, that the measured D_{22} values are close to the values of the binary diffusion coefficients at corresponding NaCl concentrations. Similarly, it is expected that the chemical potential *derivative* μ_{22} for the ternary case will be close to its corresponding binary value. Consequently, the expression for μ_{22} can be written to a good approximation as

$$\mu_{22} = \frac{RT}{C_2}\left[1 + \frac{C_2}{z_P C_1 + C_2} + 2C_2\left(\frac{\partial \ln y_2}{\partial C_2}\right)_{\text{binary}}\right] \quad (11)$$

The value of μ_{22} is evaluated using the derivative of the activity coefficient[49] for the binary salt solution at the salt concentration of the ternary system. The activity coefficient correction in eq 11 is not very sensitive to the binary concentration chosen for its evaluation. Thus, eq 11 should yield an accurate value of μ_{22}.

(iii) Calculation of μ_{12} and μ_{21}. Solving eqs 2 and 8 for the cross-derivatives of the chemical potential in terms of μ_{11}, μ_{22}, and the four $(D_{ij})_0$, we obtain

$$\mu_{12} = \{\mu_{11}[C_1\bar{V}_2(D_{22})_0 - (1 - C_1\bar{V}_1)(D_{12})_0] - \mu_{22}[C_2\bar{V}_1(D_{22})_0 - (1 - C_1\bar{V}_1)(D_{21})_0]\}\Big/ [(1 - C_2\bar{V}_2)(D_{22})_0 - (1 - C_1\bar{V}_1)(D_{11})_0] \quad (12a)$$

$$\mu_{21} = \{\mu_{11}[C_1\bar{V}_2(D_{11})_0 - (1 - C_2\bar{V}_2)(D_{12})_0] - \mu_{22}[C_2\bar{V}_1(D_{11})_0 - (1 - C_2\bar{V}_2)(D_{21})_0]\}\Big/ [(1 - C_2\bar{V}_2)(D_{22})_0 - (1 - C_1\bar{V}_1)(D_{11})_0] \quad (12b)$$

We then calculate μ_{12} and μ_{21} from the estimated values of μ_{11} and μ_{22}, and the four measured $(D_{ij})_0$ values. Numerical examination of the various terms shows that μ_{12} and μ_{21} depend mostly on μ_{22} and $(D_{21})_0$. Values of all four μ_{ij} values are given in Table 10 as μ_{ij}/RT.

If we linearly interpolate our pH 4.5 values of μ_{ij}/RT at 0.90 and 1.30 M NaCl to 1.0 M NaCl, we get $\mu_{11} = 248$ kcal mol^{-1} M^{-1} and $\mu_{12} = 6.74$ kcal mol^{-1} M^{-1}. Substituting these values into eq 8, we get $\partial\mu_1/\partial m_2 = 6.31$ kcal mol^{-1} (mol $kg^{-1})^{-1}$, in excellent agreement with the molality derivative $\partial\mu_1/\partial m_2 = (6.8 \pm 1.7)$ kcal mol^{-1} (mol $kg^{-1})^{-1}$ obtained densimetrically in equilibrium dialysis-like experiments by Arakawa and Timasheff[50] for lysozyme in buffered 1.0 M NaCl at pH 4.5 and 20 °C.

Our approach to obtaining the μ_{ij} in the ternary case can also be used for four or more components, providing that the molar concentrations of all but one solute are (a) sufficiently low that terms inversely proportional to concentration dominate the generalization of eq 9 and (b) low compared to that of the remaining solute (e.g., the supporting electrolyte). For example, consider a four-component system. In that case, the six off-diagonal μ_{ij} values are determined by three molality cross-derivatives such as eq 1, three Onsager relations similar to but more complex than eq 2, and the three main-term μ_{ii} values estimated as above.

The analysis above has been applied to the case in which one component (the protein) has a molar concentration much lower than that of the other. However, the approach can be extended to ternary systems with solutes of comparable size by extrapolating data to the limit in which one solute is infinitely dilute. Taking the limit $C_1 \to 0$ in eqs 12a and 12b yields eqs A-1 and A-2 of the Appendix. Here, D_{11}, D_{21}, and the limiting slope

$$\beta = \lim_{C_1 \to 0}(D_{12}/C_1) \quad (13)$$

are accessible by extrapolation, and D_{22} and μ_{22} are the binary

(48) Guo, H.; Kao, S.; McDonald, H.; Asanov, A.; Combs, L. L.; Wilson, W. W. *J. Cryst. Growth* **1999**, *196*, 424−433.

(49) Miller, D. G. *J. Phys. Chem.* **1966**, *70*, 2639−2659.

(50) Arakawa, T.; Timasheff, S. N. *Biochemistry* **1984**, *23*, 5912−5923.

diffusion coefficient and chemical potential derivative of component 2, respectively. Equations A-1 and A-2 have no μ_{11} approximation and apply to other cases, such as NaCl + $MgCl_2$ + H_2O.

We note that predictions of μ_{21} by the approximate "salting-out" theory of Havenga and Leaist,[27] based on our measured diffusion coefficients and the derivative of the lysozyme chloride chemical potential with respect to the lysozyme chloride concentration, are in excellent agreement with our reported values, based on the Onsager reciprocal relations. However, unlike the ORR-based approach, which is exact if the diffusion coefficients and thermodynamic data are precisely known, the salting-out theory is an approximate dilute-solution theory whose degree of validity will vary from system to system.

Use of μ_{12} and μ_{21} To Calculate the Lysozyme Cation Charge z_P. Examination of the expressions for μ_{12} and μ_{21} in eq 9 suggests that we can obtain the protein charge in our system as follows. We first multiply μ_{12}/RT and μ_{21}/RT by $z_PC_1 + C_2$ and obtain

$$Y_{12} = (z_pC_1 + C_2)\frac{\mu_{12}}{RT} = z_p + (z_pC_1 + C_2)(z_p + 1)\frac{\partial \ln y_1}{\partial C_2} \quad (14a)$$

$$Y_{21} = (z_pC_1 + C_2)\frac{\mu_{21}}{RT} = z_p + 2(z_pC_1 + C_2)\frac{\partial \ln y_2}{\partial C_1} \quad (14b)$$

We assume a linear relation $\partial \ln y_i/\partial C_j = a_{ij} + b_{ij}(z_PC_1 + C_2)$ between each activity coefficient derivative in eq 14a,b and the linear combination of concentrations (the normality N) in the same equation. We then use values of μ_{12} and μ_{21} calculated from our $C_1 = 0.60$ mM experiments at several values of C_2 to perform nonlinear least-squares fits to determine a_{12}, b_{12}, and z_P in eq 14a, and a_{21}, b_{21}, and z_P in eq 14b. The results are shown in Figure 5. (Insufficient data are available for fits using higher-order polynomial approximations to $\partial \ln y_i/\partial C_j$.) Self-consistency requires the two values of z_P to be in good agreement. At pH 4.5, the values of z_P obtained from eqs 14a and 14b are 8.83 (with $a_{12} = 0.0185$ M^{-1} and $b_{12} = 0.243$ M^{-2}) and 8.99 ($a_{21} = 8.82$ M^{-1}, $b_{21} = 2.51$ M^{-2}), respectively, while at pH 6.0 we find 7.98 ($a_{12} = -0.279$ M^{-1}, $b_{12} = 0.449$ M^{-2}) and 8.26 ($a_{21} = 7.21$ M^{-1}, $b_{21} = 3.57$ M^{-2}), respectively. This agreement suggests that our measured diffusion coefficients and estimated μ_{ij} values are consistent and reasonably accurate. The lower charge at pH 6.0 is expected, since z_P decreases to zero as the isoelectric pH of 11 is approached.

It is important to note that over our range of NaCl concentrations, we have assumed that (a) z_P is constant and (b) the linear relationships between the concentrations and the two thermodynamic derivatives in eqs 14a and 14b are valid. These assumptions are justified by the internal consistency of our results. However, values of μ_{ij} calculated on the above basis would be expected to be incorrect at very low NaCl concentrations, due to a dependence on the square root of the ionic strength.

At both pH values, values of z_P calculated from the binary experiments differ considerably from those calculated using ternary data. This is not surprising, since they were obtained using very different approaches. The averaged ternary z_P values ($8._9$ and $8._1$ at pH 4.5 and 6.0, respectively) were obtained at higher concentrations from thermodynamic data, which were in turn obtained in part from transport data. One set of binary z_P values ($6._7$ and $6._{45}$ at pH 4.5 and 6.0) calculated above was

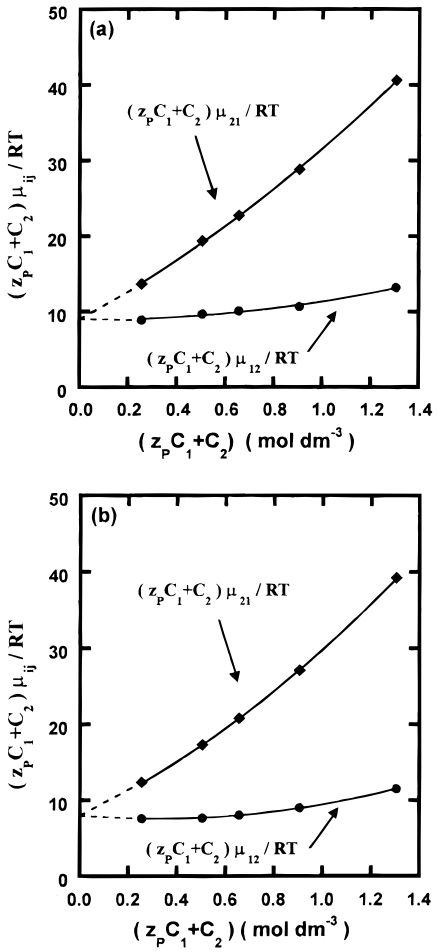

Figure 5. $(z_PC_1 + C_2)\mu_{ij}/RT$ vs $z_PC_1 + C_2$ for $i \neq j$ at 25 °C: ●, μ_{12}; ◆, μ_{21}. (a, top) pH 4.5; (b, bottom) pH 6.0. Curves fitted to data using the nonlinear least-squares procedure described in the text.

obtained from extrapolated limiting diffusion coefficients for aqueous lysozyme chloride. These limiting values were in turn extrapolated from diffusion coefficients measured at low, but nonzero concentrations. These extrapolated diffusion coefficients were used to get z_P from the binary Nernst−Hartley equation, which is an infinite-dilution transport equation. The second set of binary z_P values ($3._9$ and $4._3$ at pH 4.5 and 6.0) was obtained by a Harned-type analysis[1] in which the charge in the Debye−Hückel limiting law was adjusted to match the concentration dependence of the diffusion data. The unrealistically low values indicate the limitations of Debye−Hückel analysis in our systems.

Use of μ_{12} To Calculate the Chemical Potential Variation of Lysozyme Chloride

We divide eq 14a by $z_PC_1 + C_2$, use the linear approximation for $\partial \ln y_i/\partial C_j$ in terms of the coefficients a_{ij} and b_{ij}, and integrate to get

$$\mu_1 - \mu_1^* = \int \mu_{12}\, dC_2 = RT\left\{z_p \ln\frac{z_pC_1 + C_2}{z_pC_1 + C_{2,s}} + (z_p + 1)(C_2 - C_{2,s}) \times \left[a_{12} + b_{12}z_pC_1 + b_{12}\frac{C_2 + C_{2,s}}{2}\right]\right\} \quad (15)$$

where $C_{2,s}$ is the NaCl concentration at which 0.60 mM lysozyme chloride in aqueous NaCl is in equilibrium with

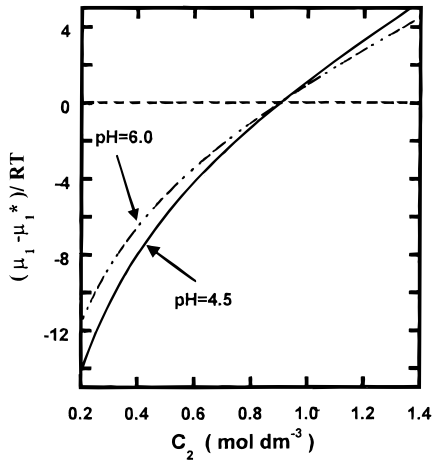

Figure 6. Integrated values of $(\mu_1 - \mu_1^*)/RT$ versus C_2: —, pH 4.5; – – •• – –, pH 6.0.

crystalline tetragonal lysozyme chloride at 25 °C. Using the values of z_P, a_{12}, and b_{12} determined from eq 14a, we can then compute the chemical potential of lysozyme chloride, μ_1, relative to the reference state (denoted by an asterisk and taken as 0.90 M at pH 4.5 and 6.0)[51] at which the lysozyme chloride activity is unity. Plots of $(\mu_1 - \mu_1^*)/RT$ versus C_2 are given in Figure 6 for pH 4.5 and 6.0.

The highest NaCl concentration that we could prepare in a bottom solution without precipitation occurring before or during an experiment was about 1.35 M. At $C_2 = 1.30$ M, the driving force for crystallization calculated from eq 15 is 10.81 kJ mol^{-1} at pH 4.5 and 9.17 kJ mol^{-1} at pH 6.0. These values depend on the uncertain value of the NaCl concentration beyond which 0.60 mM lysozyme chloride becomes insoluble (i.e., $C_{2,s} = 0.90$ M). The dependence of the uncertainty in μ_1 on the uncertainty in $C_{2,s}$ is found from

$$\frac{\partial(\mu_1 - \mu_1^*)}{\partial C_{2,s}} = -RT\left\{\frac{z_P}{z_P C_1 + C_{2,s}} + (z_P + 1)[(a_{12} + b_{12} z_P C_1) + b_{12} C_{2,s}]\right\} \quad (16)$$

and the calculated values of z_P, a_{12}, and b_{12}. At pH 6.0, each 0.1 M uncertainty in $C_{2,s}$ about the nominal 0.90 M value corresponds to an uncertainty of 2.46 kJ mol^{-1} in the driving force. For example, if $C_{2,s} = (0.90 \pm 0.05)$ M, then $\mu_1 - \mu_1^* = (9.17 \pm 1.23)$ kJ mol^{-1}. We observe that the derivative (eq 16) depends on the linear combination $z_P C_1 + C_{2,s}$, but is independent of C_2.

(51) This value is based on data at 25 °C for aqueous solutions of lysozyme and NaCl buffered with 0.05 M sodium acetate at pH 4.5, and with 0.05 M sodium phosphate at pH 6.0 (Howard, S. B.; Twigg, P. J.; Baird, J. K.; Meehan, E. J. *J. Cryst. Growth* **1988**, *90*, 94−104). Shih et al. report the only lysozyme solubility measurements in unbuffered solution near 25 °C of which we are aware (Shih, Y.-C.; Prausnitz, J. M.; Blanch, H. W. *Biotechnol. Bioeng.* **1992**, *40*, 1155−1164). However, a later paper (Curtis, R. A.; [Montaser, A.;] Prausnitz, J. M.; Blanch, H. W. *Biotechnol. Bioeng.* **1998**, *57*, 11−21; erratum **1998**, *58*, 451) reports that, for lysozyme obtained from the supplier used earlier, "molecular weights obtained from the experiments in solutions of sodium chloride are approximately 17,500 daltons, larger than the monomer molecular weight of 14,600 (*sic*) daltons indicating, that the Sigma lysozyme contains high molecular weight impurities". Curtis et al. then state that lysozyme from this supplier "contains 2% ovalbumin and conalbumin, which interact with the lysozyme to form large aggregates in aqueous salt solutions". All other solubility data known to us at or near 25 °C pertain to systems with buffer concentrations in excess of those used by Howard et al.

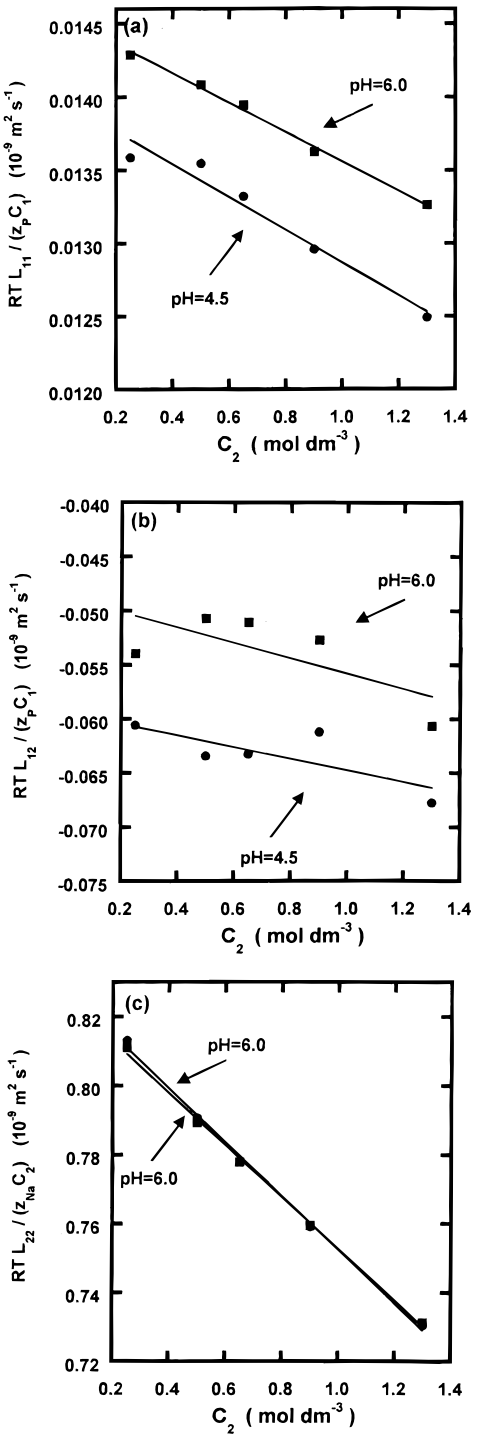

Figure 7. Normalized diffusion Onsager coefficients versus C_2 at 25 °C: ●, pH 4.5; ■, pH 6.0. (a, top) $RTL_{11}/(z_P C_1)$; (b, middle) $RTL_{12}/(z_P C_1)$; (c, bottom) $RTL_{22}/(z_{Na} C_2)$. Lines are least-squares fits.

We note that any good fit of the NaCl dependence of the values of μ_{12} derived from eq 14a could have been used to evaluate the integral in eq 15. When better ternary solubility data become available, a more accurate value of $C_{2,s}$ at 25 °C and $C_1 = 0.60$ mM can be used in eq 15 to recompute the driving force for crystallization.

Thermodynamic Transport Coefficients (Diffusion Onsager Coefficients)

The thermodynamic transport coefficients, $(L_{ij})_0$, were calculated from the $(D_{ij})_0$ and μ_{ij} data in Tables 9 and 10,

Table 11

A. Thermodynamic Transport Coefficients for pH 4.5, $C_1 = 0.60$ mM					
	$C_2 = 0.25$ M	$C_2 = 0.50$ M	$C_2 = 0.65$ M	$C_2 = 0.90$ M	$C_2 = 1.30$ M
$RTL_{11}/(z_PC_1)$ (10^{-9} m^2 s^{-1})	0.0136	0.0135	0.0133	0.0130	0.0125
$RTL_{12}/(z_PC_1)$ (10^{-9} m^2 s^{-1})	−0.061	−0.063	−0.063	−0.061	−0.068
$RTL_{22}/(z_{Na}C_2)$ (10^{-9} m^2 s^{-1})	0.813	0.791	0.778	0.759	0.730
B. Thermodynamic Transport Coefficients for pH 6.0, $C_1 = 0.60$ mM					
$C2$ (M)	$C_2 = 0.25$ M	$C_2 = 0.50$ M	0.65	0.90	1.30
$RTL_{11}/(z_PC_1)$ (10^{-9} m^2 s^{-1})	0.0143	0.0141	0.0139	0.0136	0.0133
$RTL_{12}/(z_PC_1)$ (10^{-9} m^2 s^{-1})	−0.054	−0.051	−0.051	−0.053	−0.061
$RTL_{22}/(z_{Na}C_2)$ (10^{-9} m^2 s^{-1})	0.811	0.789	0.778	0.759	0.731

respectively, and eq 7. In general, the $(L_{ij})_0$ coefficients require normalization divisors[11,47] so as not to vanish as C_1 or C_2 approaches zero. For L_{11}, L_{12} (=L_{21}), and L_{22}, these divisors are x_1N, x_1x_2N, and x_2N, respectively, where $N = z_PC_1 + C_2$ is the equivalent concentration (normality), and $x_1 = z_PC_1/(z_PC_1 + C_2)$ and $x_2 = C_2/(z_PC_1 + C_2)$ are the equivalent fractions.

In our experiments, $\bar{C}_1$ is constant and much less than $\bar{C}_2$. Values of N and x_1 are therefore approximately proportional to C_2 and z_PC_1/C_2, respectively, but x_2 will be independent of C_2 and approximately unity. The normalization factors are then $x_1N = z_PC_1$ for $(L_{11})_0$, $x_1x_2N = z_PC_1$ for $(L_{12})_0$ and $(L_{21})_0$, and $x_2N = z_{Na}C_2$ for $(L_{22})_0$. Thus, in this study, the normalization divisors for both $(L_{11})_0$ and $(L_{12})_0$ will be constant, and that for $(L_{22})_0$ will be C_2. The value of z_P used is the average of those determined from eqs 14a and 14b.

The normalized $(L_{ij})_0$ coefficients are shown in Table 11 for pH 4.5 and 6.0, and provide a basis for comparison of results at different lysozyme chloride concentrations in future experiments. Plots of the normalized quantity $(L_{22})_0/(x_2N)$ as a function of C_2 are shown in Figure 7 for pH 4.5 and 6.0. The thermodynamic transport coefficients $(L_{22})_0$ and their normalized values appear to be insensitive to pH. The normalized values depend nearly linearly on NaCl concentration. This is consistent with the common observation that descriptions of diffusive transport in terms of thermodynamic transport coefficients and chemical potential gradients better separate frictional and driving force effects than do descriptions in terms of diffusion coefficients and concentration gradients. For example, the concentration dependence of the normalized $(L_{22})_0$ is simpler than the concentration dependence of D_{22}. In the present case, one should not expect extrapolation to $C_2 = 0$ to be valid, because the chloride ions of lysozyme chloride become important at very low NaCl concentrations.

The values of $(L_{12})_0$ would be expected to decrease as the charge of the lysozyme cation decreases. The appearance of z_P in the normalization factor of $(L_{12})_0$ incompletely accounts for this effect.

Errors in μ_{11} have only small effects on μ_{12} and μ_{21}, to which the terms involving μ_{11} make only small contributions. In contrast, eq 7 shows that the relative errors in $(L_{11})_0$ and $(L_{12})_0 = (L_{21})_0$ are nearly proportional to the error in μ_{11}. However, the ratio $(L_{11})_0/(L_{12})_0$ does not depend on μ_{11} and thus is an accurate quantity.

Finally, the availability of the $(L_{ij})_0$ coefficients allows us to interpret the pH dependence of D_{21} in terms of thermodynamic quantities. From eq 6, we see that when μ_{11} is small, as is the case in our work, $(D_{21})_0$ is dominated by $(L_{22})_0\mu_{21}$. Since the first factor is close to its binary value, the main contribution to the pH dependence will come from μ_{21}. At each NaCl concentration, reference to Tables 8 and 10 above, and to Table 7 of ref 5, shows that the ratio of μ_{21} at pH 6.0 to its value at pH 4.5 (0.905, 0.893, 0.916, 0.940, and 0.968 at $C_2 = 0.25$, 0.50, 0.65, 0.90, and 1.30 M, respectively) is within 0.7% of the ratio of the diffusion coefficients $(D_{21})_0$ at the two pH values and corresponding NaCl concentrations (0.909, 0.899, 0.919, 0.944, and 0.970).

Conclusions

We have applied the Onsager reciprocal relations to precision ternary diffusion data in a fundamentally new way, to obtain two cross-derivatives of the chemical potential, $\mu_{ij} \equiv \partial\mu_i/\partial C_j$ ($i \neq j$), for aqueous protein solutions. Besides the ternary diffusion coefficients, the calculation also requires estimates of the self-derivatives μ_{ii}. Using computed values of μ_{12} and μ_{21}, we have computed approximate values for the charge of the lysozyme cation at 0.60 mM and 25 °C for pH 4.5 and 6.0 over a range of NaCl concentrations (0.25−1.30 M). Integration of μ_{12} with respect to C_2 at constant C_1 gives the change in lysozyme chloride chemical potential with NaCl concentration at fixed protein concentration well into the supersaturated region, with an apparent accuracy of 2−4%.

Many systems of biological interest satisfy the restriction that the molar concentration of protein or other monodisperse biological macromolecules is small enough for the derivative of its chemical potential with respect to its own concentration to be dominated by an inverse dependence on concentration, and also small compared to the concentration of supporting electrolyte.

The approach demonstrated here thus provides a means to obtain, relative to any reference state, the chemical potentials of proteins (with low molar concentrations) in concentrated electrolyte solutions, quantities which are typically not easily accessible. All other methods known to us either suffer from lack of accuracy when the solute of interest is dilute, give less information than the concentration variation of the chemical potential, or are inapplicable to supersaturated solutions. Our approach applies to undersaturated solutions, and to those supersaturated solutions for which the onset of precipitation occurs after the experiment ends.

The ability to determine chemical potentials (relative to a crystalline state or other reference) is of particular interest in protein crystal growth, where this technique provides, for the first time, direct access to thermodynamic variables under metastable conditions. Its detailed application to lysozyme and other biological macromolecules should provide considerable insight into nucleation, crystal growth, and related phenomena.

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Appendix

In the limit $C_1 \rightarrow 0$ (i.e., a binary solution of component 1 in the solvent), we find from eqs 10, 12a, and 12b that

$$\mu_{12} = \{RT[\bar{V}_2(D_{22})_0 - \beta] - \mu_{22}[C_2\bar{V}_1(D_{22})_0 - (D_{21})_0]\}\Big/ [(1 - \bar{V}_2C_2)(D_{22})_0 - (D_{11})_0] \quad \text{(A-1)}$$

$$\mu_{21} = \{RT[\bar{V}_2(D_{11})_0 - (1 - \bar{V}_2C_2)\beta] - \mu_{22}[C_2\bar{V}_1(D_{11})_0 - (1 - \bar{V}_2C_2)(D_{21})_0]\}\Big/ [(1 - \bar{V}_2C_2)(D_{22})_0 - (D_{11})_0] \quad \text{(A-2)}$$

where β is defined in eq 13 and the partial molar volumes are the limiting values as $C_1 \rightarrow 0$. Arbitrary numbering of components 1 and 2 in eqs 13, A-1, and A-2 allows evaluation of these activity coefficient derivatives in the limit where the concentration of either solute (but not both) vanishes.

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