

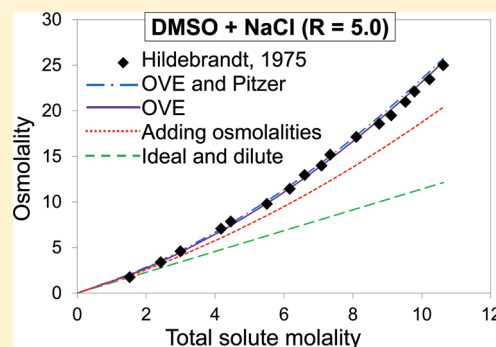
# Application of the Multisolute Osmotic Virial Equation to Solutions Containing Electrolytes

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**ABSTRACT:** The prediction of multisolute solution behavior of solutions containing electrolytes is important in many areas of research, including cryopreservation. In this study, the use of a novel form of the osmotic virial equation for multisolute solutions containing an electrolyte is investigated and compared to a rigorous electrolyte solution theory, the Pitzer–Debye–Huckel equation. For aqueous solutions containing a small molecule (either dimethyl sulfoxide or glycerol) and sodium chloride, the multisolute osmotic virial equation, which utilizes only two parameters to capture the electrolyte solution behavior, is shown to be as accurate as the Pitzer–Debye–Huckel equation, which utilizes six empirical parameters and multiple functions to capture the electrolyte solution behavior. In addition, an approach based on the multisolute osmotic virial equation to investigate the effect of electrolyte concentration on macromolecule solution behavior is presented and applied to aqueous solutions of hydroxyethyl starch and sodium chloride. The multisolute osmotic virial equation is shown to be an accurate, straightforward predictive solution theory for important multisolute solutions containing electrolytes.



## INTRODUCTION

The osmolality as a function of concentration of multisolute solutions containing electrolytes is of great importance in many areas of biology, including cryopreservation. Currently, cryopreservation is the only feasible method for long-term preservation of structure and function in natural and engineered cells and tissues for transplantation and research. As ice forms in the extracellular solution during cryopreservation, the concentration of solutes increases, creating an osmolality (or vapor pressure) difference between the intra- and extracellular solutions. Depending on the rate of cooling, the cell responds to the osmolality difference by osmotic transport of water from the intracellular solution to the extracellular solution (during slow cooling) or by freezing intracellularly (during rapid cooling). Both the exposure to concentrated solutions during slow cooling and intracellular ice formation (IIF) during rapid cooling have been shown to be damaging to cells in suspension.<sup>1–6</sup> To avoid cryo-injury, molecules called cryoprotective agents (CPAs) are added. Some CPAs such as dimethyl sulfoxide (DMSO) and glycerol permeate the cell membranes, while other CPAs such as hydroxyethyl starch (HES) are nonpermeating. The CPAs depress the thermodynamic freezing point of the intra- and extracellular solutions, thus decreasing the temperature at which ice can begin to form and how much ice will be in the solution at a given temperature. Osmolality and freezing point are related to each other, so once one is known, the other can be determined.<sup>7</sup>

Electrolyte solution behavior has been extensively studied.<sup>8–15</sup> Electrolytes are often in solution with other solutes, such as macromolecules or CPAs. One of the predominant intracellular

solutes is potassium chloride (KCl), while sodium chloride (NaCl) is found in many extracellular solutions, both in vivo (plasma) and ex vivo (phosphate buffered saline solutions). The solution behavior of these complicated multisolute solutions plays a role in the cellular response to the extracellular environment during cryopreservation. The osmolality difference between the intra- and extracellular solutions drives the water flux across the cell membrane.<sup>16,17</sup> In addition, it has been demonstrated that the osmolality as a function of concentration for the intracellular solution is needed to accurately determine the osmotically inactive fraction of the cell volume.<sup>18</sup>

To understand or predict how cells respond to a changing extracellular environment, which is brought on by the addition of CPAs or the conversion of liquid water to ice, the osmolality (or freezing point depression) as a function of concentration for these complicated multisolute solutions containing electrolytes is needed. Due to the wide range of solutes of interest and the many possible mixture compositions, measuring the solution behavior of all potential solutions is not feasible. Thus, an accurate, predictive multisolute solution theory is required.

Many multisolute solution theories have been proposed for solutions of interest in cryobiology.<sup>19–29</sup> However, many of these solution theories either (i) require fitting of the multisolute solution data and, therefore, cannot be used to predict the behavior of multisolute solutions for which there are no multisolute data

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available<sup>19,21,23–27</sup> or (ii) make assumptions regarding the interactions between solute molecules that are not thermodynamically correct for nonideal, nondilute solutions.<sup>20,22,29</sup>

Previously, we proposed a form of the multisolute osmotic virial equation that is derived from thermodynamic principles for nonideal solutions and allows prediction of multisolute solution behavior using only single-solute information.<sup>28</sup> This form of the multisolute osmotic virial equation has been shown to be applicable to a wide range of solutes and combinations of solutes.<sup>7,28,30</sup> The osmotic virial coefficients are obtained by fitting the single-solute osmotic virial equation to single-solute solution data. Using the multisolute osmotic virial equation proposed by Elliott et al.,<sup>28</sup> reasonably accurate predictions of multisolute solution behavior can be made using only single-solute coefficients.

To extend the application of the osmotic virial equation to electrolyte solutions, an additional fitting constant in the single-solute osmotic virial equation, called the “dissociation constant”, is used to capture the complicated behavior of single-solute electrolyte solutions. It can be argued that the single-solute osmotic virial equation approach should not be used to describe the solution behavior of electrolytes since (i) the dissociation constant should not be less than two for strong 1:1 electrolytes such as NaCl or KCl and (ii) a constant cannot capture the complicated electrolyte solution behavior over a range of concentrations, so an actual electrolyte solution theory, such as the Pitzer–Debye–Huckel equations,<sup>8,9</sup> should be used.

There has been much debate in the literature with respect to the question of complete or partial dissociation of NaCl<sup>31</sup> in aqueous solution. Much of the work published on NaCl is based on the assumption of complete dissociation.<sup>8,10,14,32</sup> However, others have shown that by assuming partial dissociation, equations can be developed that quantitatively explain the behavior of aqueous NaCl solutions.<sup>12,13</sup> The dissociation constant has also been shown to be dependent on electrolyte concentration, reaching complete dissociation only at infinite dilution.<sup>33</sup> Heyrovská demonstrated that by fitting equations that assume partial electrolyte dissociation to volumetric data for aqueous NaCl solutions very accurate predictions of the density could be obtained. The degree of dissociation for aqueous NaCl solutions, with concentrations ranging from 0 to 6.144 mol/kg solvent, was calculated to vary between 1 (completely dissociated) and less than 0.8.<sup>13</sup>

One of the most well-known electrolyte solution theories, the Pitzer–Debye–Huckel equation for solutions of electrolytes,<sup>8,9</sup> utilizes six empirical parameters and multiple ionic-strength-dependent functions to capture the electrolyte solution behavior, as opposed to the osmotic virial equation approach of using the dissociation constant and a second virial coefficient for a total of two fitting constants. The first purpose of this paper is to show that, when using our multisolute osmotic virial equation with our proposed mixing rule,<sup>7,28</sup> for multisolute solutions containing NaCl and a CPA (either DMSO or glycerol) using the single-solute osmotic virial equation to capture the electrolyte portion of the solution behavior results in predictions that are as accurate as using an electrolyte solution theory to capture the electrolyte portion of the solution behavior. For this purpose, the single-solute osmotic virial equation for electrolytes was compared with the Pitzer–Debye–Huckel equation for electrolytes when either was used for the electrolyte portion in the multisolute osmotic virial equation proposed by Elliott et al.<sup>7,28</sup>

In addition to demonstrating the accuracy and simplicity of the multisolute osmotic virial equation for solutions containing electrolytes, this study also investigated the use of the multisolute osmotic virial equation approach for aqueous solutions of macromolecules and electrolytes. There are many reports of macromolecular solution behavior being dependent on the ionic strength of the solution,<sup>34–37</sup> which has been attributed to changes in protein folding<sup>38</sup> and changes in the interactions between macromolecules<sup>39–44</sup>—as a result, the osmotic virial approach must be modified to allow for the effects of salt on the protein molecules. The system of hydroxyethyl starch (HES) and NaCl in water was used in this study as a model to show how the multisolute osmotic virial equation proposed by Elliott et al.<sup>28</sup> can be utilized to capture the changing macromolecular solution behavior in salt solutions. HES was chosen because it is an important solute in plasma expanders<sup>45,46</sup> and cryopreservation solutions.<sup>46–48</sup>

## THEORY

**Single-Solute Osmotic Virial Equation.** The single-solute osmotic virial equation (OVE) is applicable to a range of solutions containing water plus a single solute.<sup>7,28</sup> The osmolalities of single-solute solutions are represented as truncated polynomials in concentration, where each solute has unique coefficients for terms of second or higher order in concentration. The solute-specific coefficients are determined by fitting the single-solute OVE to single-solute data for osmolality as a function of solute concentration.

Written as a function of solute molality,  $m_i$ , the single-solute OVE is

$$\pi = m_i + B_i m_i^2 + C_i m_i^3 + \dots \quad (1)$$

where  $\pi$  is the osmolality of the solution (osmoles/kg solvent);  $m_i$  is the molal concentration of the solute (moles solute/kg solvent); and  $B_i$  [(moles solute/kg solvent)<sup>−1</sup>] and  $C_i$  [(moles solute/kg solvent)<sup>−2</sup>] are the second and third osmotic virial coefficients for use with molality, respectively. This equation can also be written in terms of mole fraction, which requires one additional conversion factor between osmolality and osmole fraction ( $\tilde{\pi}$ , osmoles/mole solution).<sup>7</sup>

The osmotic virial coefficients can be derived from knowledge of the interactions between solute molecules, where the second virial coefficient represents interactions between two solute molecules, the third virial coefficient represents interactions between three solute molecules, and so on.<sup>49</sup> The single-solute OVE may be truncated after a small number of terms that are sufficient to accurately describe the solution behavior over the concentration range of interest. Previously, we have shown that the adequacy of fit can be determined using methods such as the adjusted  $R^2$  criterion.<sup>7,50</sup>

For electrolytes, one additional single-solute fitting parameter is proposed. In similar equations, this parameter has been referred to as the “dissociation constant” ( $k_{\text{diss}}$ ) but has also been known as the van’t Hoff factor.

$$\pi = k_{\text{diss}} m_i + B_i (k_{\text{diss}} m_i)^2 + C_i (k_{\text{diss}} m_i)^3 + \dots \quad (2)$$

The dissociation constant accounts for the additional nonideality of the solution behavior from several electrolyte effects, meaning that the empirically determined constant may not be exactly equal to the sum of the stoichiometric number of ions.

**Table 1. Osmotic Virial Equation Parameters for Use with Solution Molality**

solute	$k_{\text{diss}}$ [ $\pm 95\%$ CI]	$B$ (molal <sup>-1</sup> ) [ $\pm 95\%$ CI] <sup>a,b</sup>	adj. $R^2$
NaCl <sup>c</sup>	1.673 [ $\pm 0.02$ ]	0.0508 [ $\pm 0.002$ ]	1.000
DMSO <sup>d</sup>	1	0.108 [ $\pm 0.005$ ]	0.990
glycerol <sup>e</sup>	1	0.023 [ $\pm 0.001$ ]	0.996

<sup>a</sup> 95% confidence intervals were calculated using the methods outlined previously.<sup>7</sup> <sup>b</sup> All of the solutes in Table 1 were adequately represented by a quadratic fit (i.e., osmotic virial coefficients greater than the second order were not required). <sup>c</sup> The values for the NaCl osmotic virial coefficients used in the current study are slightly different from those previously published.<sup>7</sup> This is because a different data set from the literature was used to obtain the osmotic virial coefficients. In the previous study,<sup>7</sup> freezing point depression data<sup>61</sup> were used because therein we used the single-solute coefficients to predict the freezing point of multisolute solutions. In the current study, we used the same data set<sup>14</sup> as was used by Pitzer et al.<sup>9</sup> to determine their NaCl parameters. <sup>d</sup> Data from references 28, 58, 60, and 62. <sup>e</sup> Data from references 28, 58, 59, and 61.

In other studies,<sup>12</sup> the van't Hoff factor,  $i$  (defined as the number of ions that the molecule dissociates into), has been calculated using

$$m[(1-d) + 2d] = im \quad (3)$$

where  $d$  = the degree of dissociation and  $m$  is the molal concentration of the electrolyte. The degree of dissociation ( $d$ ) for aqueous solutions of NaCl with concentrations ranging from 0 to 6.144 mol/kg of solvent has been shown to vary between 1 (completely dissociated) and less than 0.8.<sup>13</sup> From eq 3, this results in a van't Hoff factor ranging from 2 ( $d = 1$ ) to less than 1.8 ( $d < 0.8$ ). While the van't Hoff factor is understood to be the number of ions into which a molecule dissociates, the inaptly named "dissociation constant" should merely be considered as an empirical fitting constant in eq 2.

In this study, the osmotic virial coefficients for the CPAs (glycerol and DMSO) were taken from prior results.<sup>7</sup> The osmotic virial coefficients and the dissociation constant for NaCl were determined by fitting eq 2 to data from the literature.<sup>14</sup> All of the coefficients utilized are listed in Table 1. A more comprehensive list of osmotic virial coefficients for a wide range of solutes has been previously published.<sup>7</sup>

**Pitzer–Debye–Huckel Electrolyte Solution Theory.** Many other electrolyte solution theories have been developed to describe the complex solution behavior of electrolytes in water. Among the most well-known is the Pitzer–Debye–Huckel electrolyte solution theory.<sup>8,9</sup> Pitzer and co-workers incorporated the Debye–Huckel equations into a solution theory to describe the behavior of a wide range of electrolyte solutions. In addition to being widely used, the Pitzer–Debye–Huckel equation follows the form of the OVE, making it possible to incorporate the Pitzer–Debye–Huckel equation into the form of the multisolute OVE we are proposing. Unlike the single-solute OVE, the Pitzer–Debye–Huckel equation uses a function,  $f^\Phi$ , to account for the electrolyte solution behavior.<sup>8,9</sup> This function depends on the ionic strength of the solution and thus has to be calculated for each electrolyte concentration. The Pitzer–Debye–Huckel equation in terms of osmotic coefficient,  $\Phi$ , for 1:1 electrolytes such as NaCl or KCl is<sup>9</sup>

$$\Phi - 1 = f^\Phi + B_{\text{MX}}^\Phi m + C_{\text{MX}}^\Phi m^2 \quad (4)$$

**Table 2. Pitzer–Debye–Huckel Equation Parameters**

parameter	value
$f^\Phi$	Pitzer–Debye–Huckel electrolyte effects function $f^\Phi = -A^\Phi \left( \frac{\sqrt{I}}{1 + b\sqrt{I}} \right)$
$A^\Phi$	Debye–Huckel slope <sup>a</sup> 0.391
$I$	solution ionic strength (mol/kg solvent) <sup>b</sup> $I = \frac{1}{2}(z_m^2 m + z_x^2 m)$
$b$	empirical parameter 1.2
$B_{\text{MX}}^\Phi$	2nd virial coefficient $B_{\text{MX}}^\Phi = \beta_{\text{MX}}^{(0)} + \beta_{\text{MX}}^{(1)} e^{-\alpha\sqrt{I}}$
$\beta_{\text{MX}}^{(0)}$	0.0765
$\beta_{\text{MX}}^{(1)}$	0.2664
$\alpha$	empirical parameter 2.0
$C_{\text{MX}}^\Phi$	3rd virial coefficient 0.00127

<sup>a</sup> Value for  $A^\Phi$  is for water at 25 °C and saturated pressure.<sup>51</sup> All other values can be found in reference 9. <sup>b</sup>  $z_m$  = charge of the positive ion,  $z_x$  = charge of the negative ion,  $m$  = molality of the solute. For a 1:1 electrolyte  $I = m$ .

where  $B_{\text{MX}}^\Phi$  is the second osmotic virial coefficient;  $C_{\text{MX}}^\Phi$  is the third osmotic virial coefficient;  $f^\Phi$  is the "electrolyte effects" function based on Debye–Huckel parameters; and  $m$  is the molality of the electrolyte.

To convert osmotic coefficient to osmolality for solutions containing electrolytes, the number of moles that the electrolyte dissociates into ( $\nu$ ) has to be taken into account<sup>12</sup>

$$\Phi = \frac{\pi}{\nu m} \quad (5)$$

Converting osmotic coefficient to osmolality using eq 5, and noting for NaCl that  $\nu = 2$ , gives

$$\pi = 2(f^\Phi + 1)m + 2B_{\text{MX}}^\Phi m^2 + 2C_{\text{MX}}^\Phi m^3 \quad (6)$$

In addition to the electrolyte effects function, the second osmotic virial coefficient,  $B_{\text{MX}}^\Phi$ , is also dependent on the ionic strength of the solution and thus must be calculated at each electrolyte concentration.

The electrolyte effects function is calculated using the Debye–Huckel slope,  $A^\Phi$ , the ionic strength of the solution,  $I$ , and an empirical parameter,  $b$ .<sup>8,9</sup>

$$f^\Phi = -A^\Phi \left( \frac{\sqrt{I}}{1 + b\sqrt{I}} \right) \quad (7)$$

The values for all of the empirical parameters in the Pitzer–Debye–Huckel equation are listed in Table 2.<sup>9,51</sup>

The second virial coefficient is calculated based on three empirical parameters,  $\beta_{\text{MX}}^{(0)}$ ,  $\beta_{\text{MX}}^{(1)}$ , and  $\alpha$ , and on the ionic strength of the solution<sup>8,9</sup>

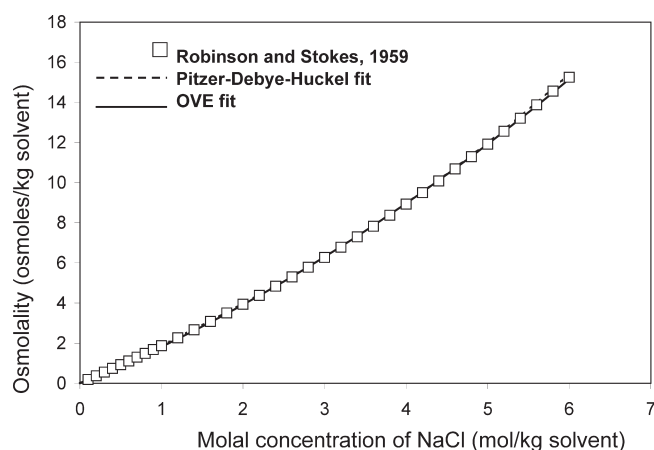
$$B_{\text{MX}}^\Phi = \beta_{\text{MX}}^{(0)} + \beta_{\text{MX}}^{(1)} e^{-\alpha\sqrt{I}} \quad (8)$$

The third virial coefficient,  $C_{\text{MX}}^\Phi$ , is a constant, which was obtained by fitting the Pitzer–Debye–Huckel equation to single-solute NaCl data.<sup>9</sup>

$$C_{\text{MX}}^\Phi = 0.00127 \quad (9)$$

Figure 1 demonstrates that the single-solute OVE (eq 2) and the Pitzer–Debye–Huckel equations (eqs 6–9) accurately capture the behavior of single-solute aqueous NaCl solutions over a wide





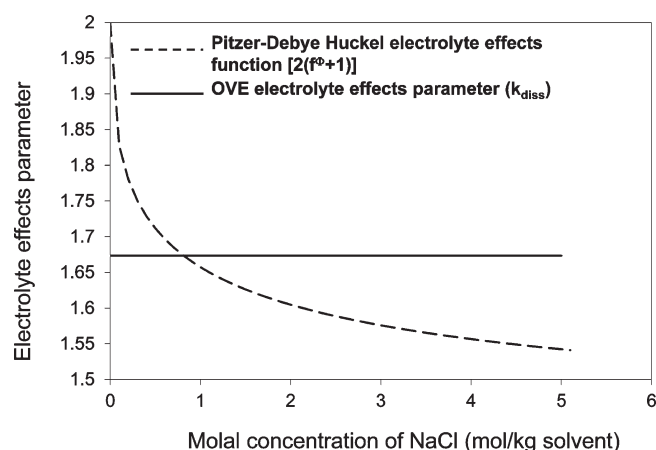
**Figure 1.** Osmolality as a function of solute molality for an aqueous single-solute NaCl solution. The open squares are data from Robinson and Stokes,<sup>14</sup> which were used by Pitzer and Mayorga<sup>9</sup> to determine the Pitzer–Debye–Huckel coefficients and were used in this study to obtain the single-solute NaCl osmotic virial coefficients. The solid line is the single-solute osmotic virial equation (eq 2) fit to the data, and the dashed line is the Pitzer–Debye–Huckel equation (eq 6) fit to the data. The Pitzer–Debye–Huckel fit lies directly under the osmotic virial equation fit over most of the concentration range and is thus not visible except at the highest NaCl concentrations.

concentration range. The application of the OVE to electrolyte solutions is straightforward, requiring only two fitting parameters to describe the solution behavior of NaCl (the dissociation constant,  $k_{\text{diss}}$ , and a second virial coefficient,  $B$ ). Alternatively, the Pitzer–Debye–Huckel equation contains more complexity—six empirical parameters and multiple functions that are ionic strength dependent are required.<sup>8,9</sup>

A comparison between the dissociation constant ( $k_{\text{diss}}$ ) and the Pitzer–Debye–Huckel electrolyte effects function ( $2(f^{\text{P}} + 1)$ ) for NaCl is shown in Figure 2. From this figure it can be seen that the value obtained by fitting for  $k_{\text{diss}}$  (constrained to be constant) is within the range of values for the  $2(f^{\text{P}} + 1)$  function calculated over a range of NaCl concentrations. While both the single-solute OVE and the Pitzer–Debye–Huckel equation adequately capture the solution behavior of an aqueous NaCl solution (Figure 1), the single solute OVE does so with a constant  $k_{\text{diss}}$ , and the Pitzer–Debye–Huckel equation does so with a function  $2(f^{\text{P}} + 1)$ .

The single-solute OVE [eqs 1 and 2] and the Pitzer–Debye–Huckel solution theory presented above [eqs 4–9] are for solutions containing water plus one solute. The Pitzer–Debye–Huckel solution theory is for electrolyte solutes, whereas the single-solute OVE can be applied to a wide variety of solutes, from electrolytes to CPAs to macromolecules. In applying either of these solution theories, the single-solute solution equations are used to obtain solute-specific coefficients (such as the parameters appearing in the Pitzer–Debye–Huckel equations or the dissociation constant and virial coefficient appearing in the osmotic virial equation), by fitting to single-solute solution data. Since most solutions of interest in biology contain multiple solutes, solution theories that can be used to predict multisolute solution behavior are required, preferably without the need to fit multisolute solution data.

**Multisolute Osmotic Virial Equation.** Various methods have been proposed to predict solution behavior of aqueous solutions containing multiple solutes of interest to cryobiology. Many of



**Figure 2.** Comparison between the dissociation constant ( $k_{\text{diss}}$ ) and the Pitzer–Debye–Huckel electrolyte effects function ( $2(f^{\text{P}} + 1)$ ) for NaCl.

these approaches require either (i) fitting of multisolute data to obtain empirical parameters<sup>19,23–27,30</sup> or (ii) overly simplifying assumptions regarding the interactions between solute molecules.<sup>29</sup>

To address these limitations, Elliott et al. proposed a novel form of the multisolute OVE with simple mixing rules. As has been shown previously,<sup>28</sup> this form of the multisolute OVE is a solution theory derived from thermodynamic principles that takes into account solute–solute interactions and allows predictions of multisolute solutions without the need to fit multisolute data.<sup>7,28</sup> The form of the multisolute osmotic virial equation proposed by Elliott et al.<sup>28</sup> has been shown to be accurate for a wide range of combinations of solutes over large concentration ranges.<sup>7,28,30</sup>

The form of the multisolute OVE proposed by Elliott et al.<sup>28</sup> is

$$\pi = \sum_i m_i + \sum_i \sum_j \frac{(B_i + B_j)}{2} m_i m_j + \sum_i \sum_j \sum_k (C_i C_j C_k)^{1/3} m_i m_j m_k \quad (10)$$

where the subscripts  $i, j, k$  refer to the individual solutes.

Equation 10 predicts multisolute solution behavior based only on the single-solute osmotic virial coefficients ( $B_i, C_i$ ). For electrolytes, we propose that the molality of the electrolyte be multiplied by the dissociation constant throughout eq 10. Unlike the virial coefficient mixing rules, the inclusion of the dissociation constant with the electrolyte molality was not derived from first principles but is an assumption made by the authors.

Written for an aqueous solution containing one electrolyte solute (subscript 2) and one nonelectrolyte solute (subscript 3), eq 10 is thus

$$\pi = k_{\text{diss}} m_2 + m_3 + B_2 (k_{\text{diss}} m_2)^2 + B_3 m_3^2 + (B_2 + B_3) k_{\text{diss}} m_2 m_3 + C_2 (k_{\text{diss}} m_2)^3 + C_3 m_3^3 + (C_2 C_3)^{1/3} (k_{\text{diss}} m_2)^2 m_3 + (C_2 C_3)^{1/3} k_{\text{diss}} m_2 m_3^2 \quad (11)$$

(The subscript 1 is usually reserved for the solvent, water.)

For small molecules, such as many CPAs (subscript 3) and electrolytes (subscript 2), a third virial coefficient is typically not required to describe the single-solute solution behavior, so the cubic terms in eq 11 may be set to zero, giving

$$\pi = k_{\text{diss}} m_2 + m_3 + B_2 (k_{\text{diss}} m_2)^2 + B_3 m_3^2 + (B_2 + B_3) k_{\text{diss}} m_2 m_3 \quad (12)$$

However, macromolecules often require a third virial coefficient to describe the nonideality of their solution behavior. Thus, solutions containing a macromolecule (subscript 3) and electrolyte (subscript 2) will contain the  $C_3m_3^3$  term, but the cubic mixing terms will still be equal to zero (provided the electrolyte does not have a third virial coefficient)

$$\pi = k_{\text{diss}}m_2 + m_3 + B_2(k_{\text{diss}}m_2)^2 + B_3m_3^2 + (B_2 + B_3)k_{\text{diss}}m_2m_3 + C_3m_3^3 \quad (13)$$

The multisolute OVE (10) has been used to make predictions for solutions with combinations of nonelectrolyte solutes using only single-solute information. Equation 12 predicts the solution behavior of a multisolute aqueous solution containing an electrolyte and a small molecule. Equation 13 can be used for aqueous solutions containing an electrolyte and a macromolecule.

**Combination of the Multisolute Osmotic Virial Equation with the Pitzer–Debye–Huckel Equation.** In eqs 10–13, the contribution of the electrolyte to the multisolute solution osmolality is predicted using the osmotic virial coefficients and a dissociation constant determined from fitting single-solute electrolyte solution data. However, it can be argued that the predictions from the multisolute OVE for the solution behavior of an aqueous solution containing an electrolyte and a nonelectrolyte could be improved by replacing, for the electrolyte portion of the solution behavior, the single-solute OVE approach with an electrolyte solution theory, such as the Pitzer–Debye–Huckel equation. The purpose of this study is to compare the predictions from the multisolute OVE of the osmolality of aqueous multisolute solutions containing NaCl with a CPA (either DMSO or glycerol), using either the OVE or the Pitzer–Debye–Huckel solution theory to calculate the contribution of the electrolyte to the total solution osmolality. The multisolute OVE was used to calculate the contribution of the nonelectrolyte solute and to calculate the contribution of the interactions between the two different solutes. To combine the Pitzer–Debye–Huckel with the multisolute OVE, the virial coefficients from the Pitzer–Debye–Huckel equation had to be put in the same form as the osmotic virial coefficients in the multisolute OVE so that they could be used in the multisolute OVE mixing rule.

In the single-solute OVE for electrolytes, eq 2, it can be seen that the molality of the electrolyte is always multiplied by the parameter,  $k_{\text{diss}}$ . In the Pitzer–Debye–Huckel equation for a single electrolyte in solution, eq 6, it can be seen that the function,  $2(f^\Phi + 1)$ , only appears in the linear term. Comparing the quadratic terms of the Pitzer–Debye–Huckel equation (eq 6) and the OVE (eq 2) for a single electrolyte solute shows

$$2B_{\text{MX}}^\Phi = B_i k_{\text{diss}}^2 \quad (14)$$

Comparing the linear terms shows

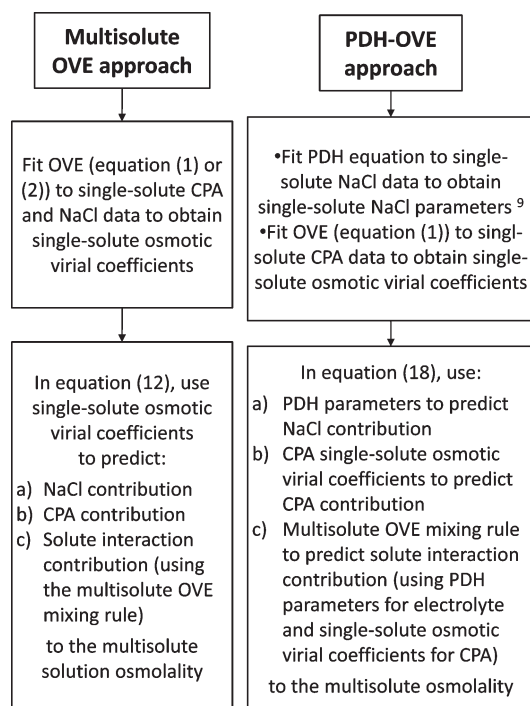
$$k_{\text{diss}} = 2(f^\Phi + 1) \quad (15)$$

Combining eqs 14 and 15 gives

$$B_i = \frac{2B_{\text{MX}}^\Phi}{[2(f^\Phi + 1)]^2} \quad (16)$$

Thus, the mixing rule for the OVE becomes

$$B_{23} = \frac{\left( B_3 + \frac{2B_{\text{MX}}^\Phi}{[2(f^\Phi + 1)]^2} \right)}{2} \quad (17)$$



**Figure 3.** Comparison of the two approaches used to predict the multisolute CPA + NaCl solution behavior: the multisolute osmotic virial equation (OVE) and the combination of the Pitzer–Debye–Huckel equation with the multisolute OVE (PDH–OVE).

Using eqs 15–17 to incorporate Pitzer–Debye–Huckel electrolyte contributions into the multisolute OVE for an electrolyte and CPA, eq 12, gives

$$\pi = [2(f^\Phi + 1)]m_2 + m_3 + 2B_{\text{MX}}^\Phi m_2^2 + B_3m_3^2 + \left( B_3 + \frac{2B_{\text{MX}}^\Phi}{[2(f^\Phi + 1)]^2} \right)[2(f^\Phi + 1)]m_2m_3 \quad (18)$$

Equation 18 can be compared to eq 12 to determine if using an electrolyte solution theory (the Pitzer–Debye–Huckel equation with  $2(f^\Phi + 1)$  and  $B_{\text{MX}}^\Phi$ ) to determine the electrolyte contribution to the osmolality of a multisolute solution is more accurate than using the OVE (with  $k_{\text{diss}}$  and  $B_i$ ) to determine the electrolyte contribution to the total solution osmolality. In both approaches (eqs 12 and 18), the OVE is used to determine the contribution of the nonelectrolyte solute, and the mixing rule for the multisolute OVE proposed by Elliott et al.<sup>28</sup> is used to determine the contribution of the interactions between the electrolyte and nonelectrolyte solute. Equation 18 will be referred to herein as the PDH–OVE approach, and eq 12 is referred to as the multisolute OVE approach. Figure 3 is a flowchart that illustrates the two approaches.

#### Using the Multisolute Osmotic Virial Equation to Investigate Electrolyte Effects on Macromolecule Solution Behavior.

All of the work presented previously on this form of the multisolute OVE, eq 10, has been using information from single solutes to make predictions of multisolute solution behavior.<sup>7,18,28,30,52–56</sup>

However, in some cases only multisolute solution data are available. For example, most macromolecule solution behavior is measured in electrolyte solutions of varying ionic strength.<sup>35–37,57</sup>

The electrolyte concentration of the solution may have a marked

effect on macromolecule solution behavior.<sup>34,36,38</sup> In this study, the multisolute OVE approach was adapted to determine the single-solute osmotic virial coefficients of a macromolecule (HES) from multisolute data of HES in varying ionic strength solutions of NaCl. This approach allows the effect of electrolyte concentration on the solution behavior of a macromolecule to be studied by investigating the changing single-solute macromolecular osmotic virial coefficients as a function of electrolyte concentration. To determine the single-solute osmotic virial coefficients, the multisolute OVE for a macromolecule and an electrolyte (eq 13) was rearranged such that the quantities that are known are on the

left-hand side of the equation and the unknown quantities on the right-hand side of the equation. Rearranging eq 13 for an aqueous solution of a macromolecule and electrolyte (where the molality of both solutes and the osmotic virial coefficients of the electrolyte are known) gives

$$\begin{aligned} \pi - k_{\text{diss}}m_2 - m_3 - B_2(k_{\text{diss}}m_2)^2 - B_2k_{\text{diss}}m_2m_3 \\ = B_3(m_3^2 + k_{\text{diss}}m_2m_3) + C_3m_3^3 \end{aligned} \quad (19)$$

The osmotic virial coefficients of HES,  $B_3$  and  $C_3$ , were determined by fitting eq 19 to multisolute osmolality as a function of

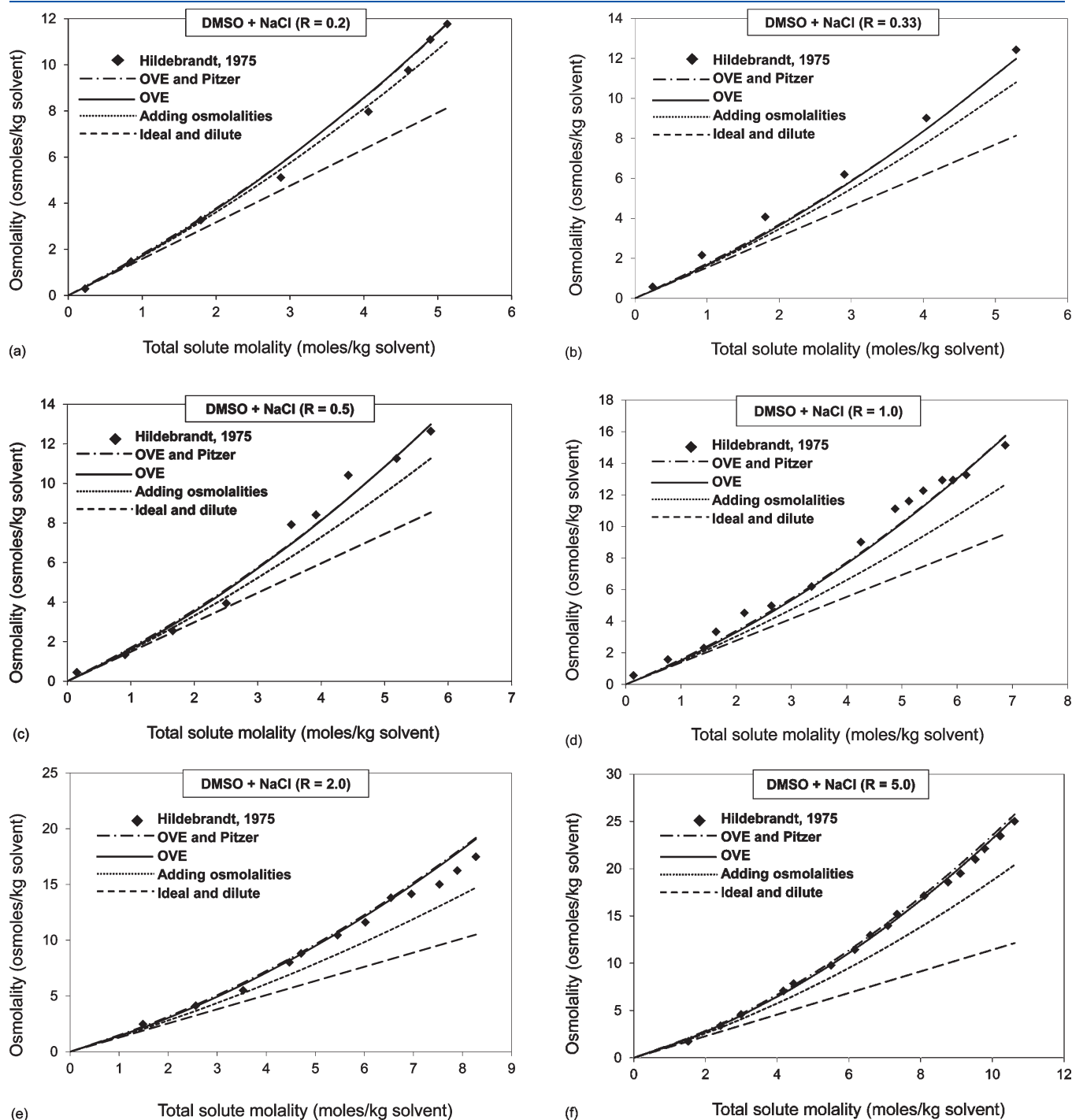
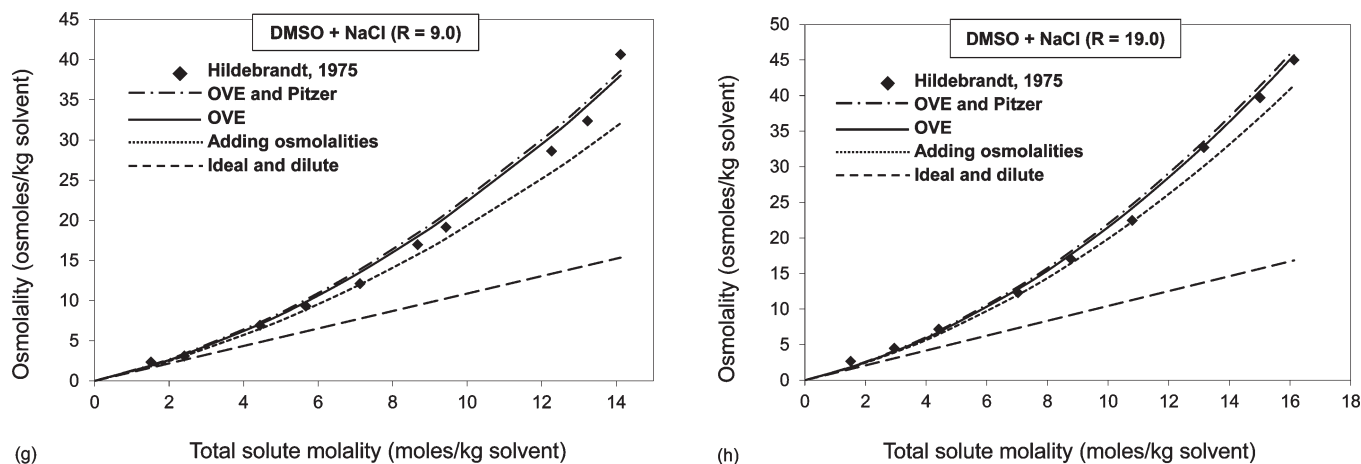


Figure 4. Continued



**Figure 4.** (a–h) Osmolality as a function of total solute molality for DMSO + NaCl + water solutions ( $R$  ranging from 0.2 to 19.0). The closed diamonds are data from Hildebrandt.<sup>62</sup> The solid line is the prediction from the multisolute OVE equation, eq 12. The long-and-short-dashed line is the prediction from the PDH–OVE equation, eq 18. The short-dashed line is the prediction from the adding-osmolalities approach, eq 21. The long-dashed line is the prediction from assuming an ideal, dilute solution, eq 22. The OVE and PDH–OVE predictions lie directly over each other in all panels where both cannot be seen.

concentration data using linear regression. This was done in a range of NaCl concentrations to determine  $B_3$  and  $C_3$  as a function of NaCl concentration.

## METHODS

Phase diagrams were obtained from the literature for single-solute DMSO, glycerol, and NaCl in water solutions.<sup>28,58–62</sup> The phase diagrams are either given as freezing point depression as a function of solute concentration or as osmolality as a function of solute concentration. The relationship between osmolality,  $\pi$ , and freezing point depression,  $\Delta T_{FP}$ , can be obtained from the Gibbs–Duhem equation<sup>7,63</sup>

$$\pi = \frac{T_{FP}^0 - T_{FP}}{[W_1 / (s_1^{0L} - s_1^{0S})] RT_{FP}} \quad (20)$$

where  $\pi$  is the osmolality of the solution (osmol/kg solvent);  $T_{FP}^0$  is the freezing point of the pure solvent (i.e., water) (273.15 K);  $T_{FP}$  is the freezing point of the solution (K);  $W_1$  is the molecular weight of water ( $1.802 \times 10^{-3}$  kg/mol);  $s_1^{0L}$  and  $s_1^{0S}$  are the entropies per mole of pure water in the liquid phase and pure water in the solid phase, respectively, and  $R$  is the universal gas constant (8.314 J/mol K). In the derivation of eq 20, the molar entropies of water,  $s_1^{0L}$  and  $s_1^{0S}$ , are assumed to be constant and  $(s_1^{0L} - s_1^{0S})$  to be equal to 22.00 J/mol.

The osmotic virial coefficients were determined for each CPA or electrolyte by fitting the single-solute OVE, eq 1 or 2, to the single-solute data. The details of the fitting procedure have been previously published.<sup>7</sup>

The multisolute solution data for aqueous solutions of HES and NaCl were obtained from the literature.<sup>57</sup> The freezing point as a function of total solute concentration was measured for various  $R$  values of HES in NaCl using differential scanning calorimetry (DSC), where  $R$  is the mass ratio of HES to NaCl ( $R = \text{mass HES}/\text{mass NaCl}$ ). For cryobiological applications,  $R$  values are often chosen to express the solution composition since as the solution freezes pure water is removed as ice, so the solutes become concentrated but the mass ratio of the two solutes

remains constant. Jochem and Korber measured the phase diagrams for HES and NaCl in water for  $R$  values ranging from 0.5 to 20.<sup>57</sup>

To determine the osmotic virial coefficients for HES in the different NaCl solutions, linear regression was performed using Excel (Microsoft, Redmond, WA, USA) using the matrix method for linear regression.<sup>50</sup> The confidence intervals were also calculated at various levels of significance. A detailed description of the linear regression procedure utilized to obtain the osmotic virial coefficients and the statistical analysis done on the coefficients has been previously published.<sup>7</sup>

## RESULTS

**CPA + NaCl + Water Solutions.** The multisolute OVE for an electrolyte and small molecule, eq 12, and the PDH–OVE approach, eq 18, were both used to predict the solution behavior of a CPA and NaCl in water. Two different CPAs were chosen, DMSO and glycerol, and a range of  $R$  values for each multisolute solution studied. The results for DMSO + NaCl + water can be found in Figure 4(a–h), for  $R$  values ranging from 0.2 to 19.0. The results for glycerol + NaCl + water can be found in Figure 5(a–g) for  $R$  values ranging from 0.25 to 9.0. In addition to the OVE approach and the PDH–OVE approach, two other predictions are also shown in the figures. The solution behaviors of the CPA + NaCl in water solutions were predicted by adding the osmolalities of the single-solute solutions, which requires the same amount of information as the multisolute OVE, eq 12, but neglects the mixing term.

$$\pi = k_{\text{diss}} m_2 + m_3 + B_2 (k_{\text{diss}} m_2)^2 + B_3 m_3^2 \quad (21)$$

This approach is equivalent to the Kleinhans and Mazur approach of adding freezing point depressions.<sup>29</sup> In Figures 4 and 5, single-solute osmotic virial coefficients from Table 1 were utilized in the adding-osmolalities predictions, not the Kleinhans and Mazur coefficients, so that the predictions from the adding-osmolalities approach and the multisolute OVE approach utilize the same coefficients.

The fourth prediction shown in Figures 4 and 5 is obtained by assuming that the mixtures of CPA and NaCl are ideal, dilute solutions. This approach assumes that none of the solute



molecules are interacting with each other (i.e., all of the osmotic virial coefficients are equal to zero) and thus the osmolality is equal to the sum of the solute molalities. The dissociation constant for the electrolyte is still taken into account. Thus, the ideal, dilute solution osmolality is given by

$$\pi = k_{\text{diss}}m_2 + m_3 \quad (22)$$

The error in the predictions can be quantified by calculating the percent error at the maximum measured solute molality and the sum of squared errors (SSE) over the entire concentration range.

The percent error is calculated using

$$\% \text{ error} = \frac{|\text{Prediction} - \text{Measured}|}{\text{Measured}} \times 100 \quad (23)$$

The sum of squared errors is calculated using

$$\text{SSE} = \sum_m (y_i - f_i)^2 \quad (24)$$

where  $y_i$  is the measured osmolality for the  $i$ th data point;  $f_i$  is the predicted osmolality for the  $i$ th data point; and  $m$  is the total

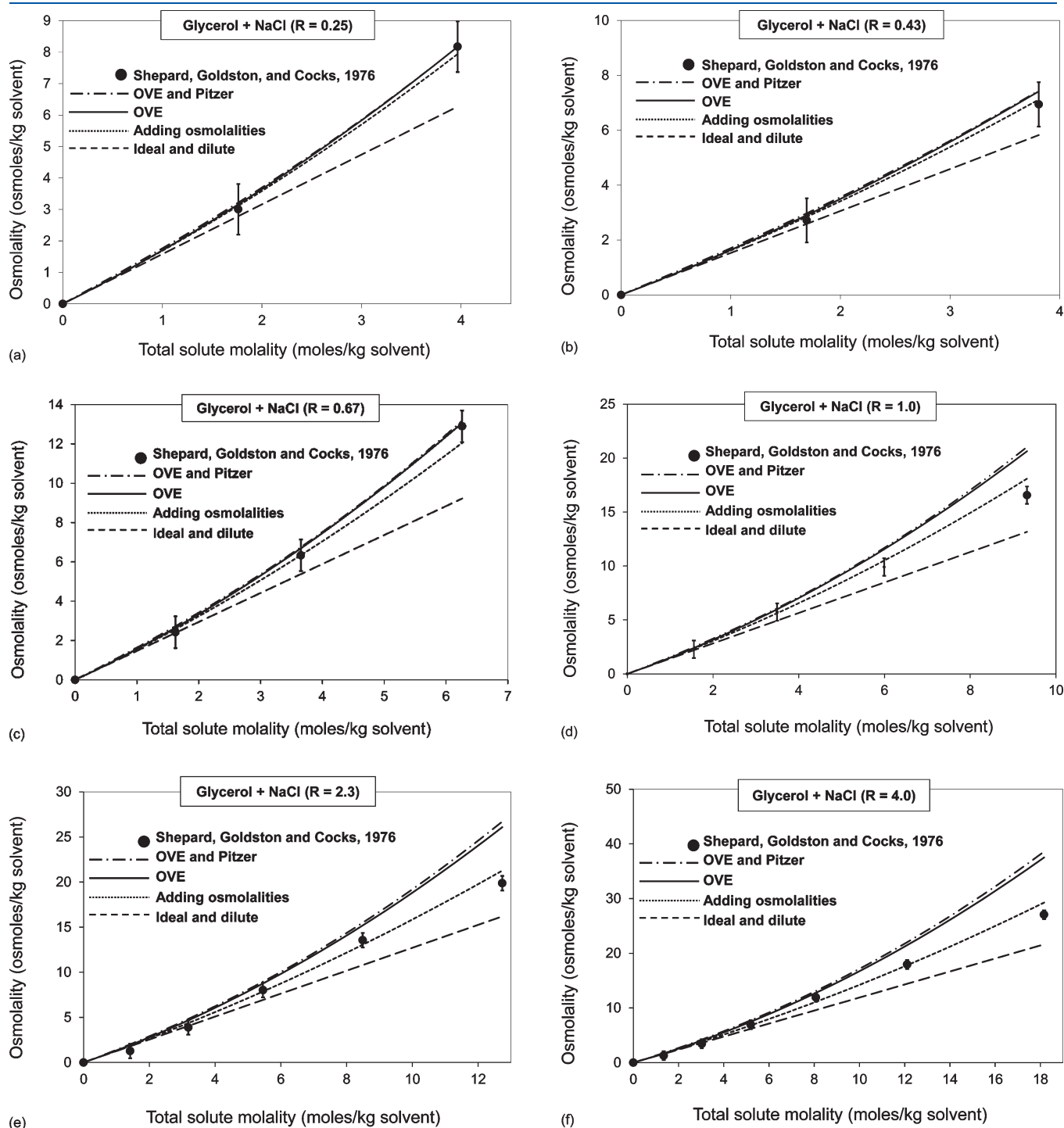
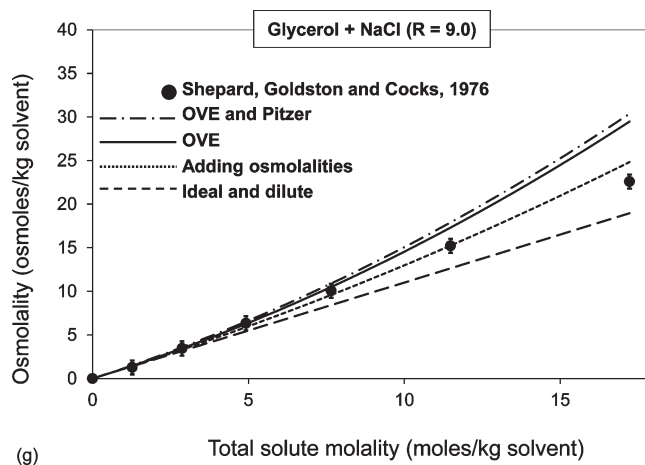


Figure 5. Continued



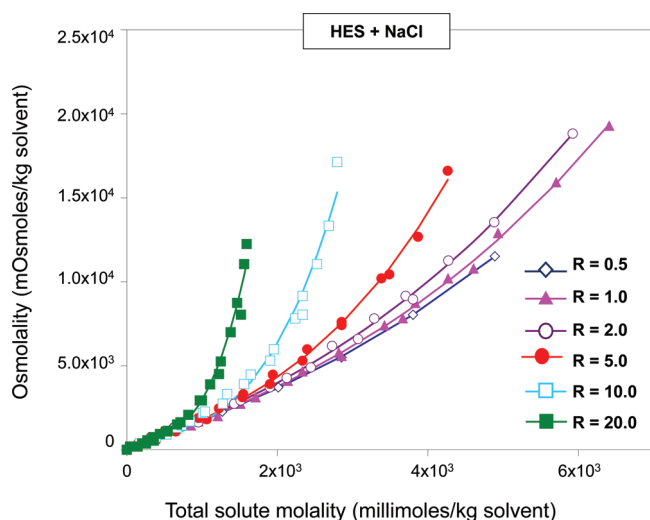


**Figure 5.** (a–g) Osmolality as a function of total solute molality for glycerol + NaCl + water solutions ( $R$  ranging from 0.25 to 9.0). The closed circles are data from Shephard et al.<sup>64</sup> The solid line is the prediction from the multisolute OVE equation, eq 12. The long-and-short-dashed line is the prediction from the PDH-OVE equation, eq 18. The short-dashed line is the prediction from the adding-osmolalities approach, eq 21. The long-dashed line is the prediction from assuming an ideal, dilute solution, eq 22. The OVE and PDH-OVE predictions lie directly over each other in all panels where both cannot be seen.

**Table 3.** Percent Errors in the Predictions from the Multisolute Solution Theories That Do Not Require Fitting of Multisolute Data

solutes [ $R$ value] <sup>a</sup>	max molality	ideal, dilute		adding osmolalities		multisolute OVE		PDH–OVE	
		% error at max molality <sup>b</sup>	SSE <sup>c</sup>	% error at max molality <sup>b</sup>	SSE <sup>c</sup>	% error at max molality <sup>b</sup>	SSE <sup>c</sup>	% error at max molality <sup>b</sup>	SSE <sup>c</sup>
DMSO + NaCl [ $R = 0.2$ ] <sup>d</sup>	5.1	30.9%	$1.3 \times 10^2$	6.4%	1.3	0.3%	1.2	0.1%	1.2
DMSO + NaCl [ $R = 19.0$ ] <sup>d</sup>	16.1	62.6%	$2.1 \times 10^3$	7.9%	28.8	1.5%	6.2	3.3%	13.6
Glycerol + NaCl [ $R = 0.67$ ] <sup>e</sup>	6.3	28.5%	14.4	6.5%	0.7	1.3%	0.2	2.1%	0.3
Glycerol + NaCl [ $R = 9.0$ ] <sup>e</sup>	17.2	16.1%	23.5	9.9%	5.5	30.5%	52.0	34.4%	68.4

<sup>a</sup>  $R$  values are the mass ratios:  $R = (\text{Mass of solute 1}/\text{Mass of solute 2})$ . <sup>b</sup> Percent error calculated using eq 23 at the maximum total solute molality at which osmolality was measured for each solution. <sup>c</sup> SSE calculated using equation 24. The values of the SSE should only be compared for the different predictions for each specific solution, not between solutions. <sup>d</sup> Data from reference 62. <sup>e</sup> Data from reference 64.



**Figure 6.** Osmolality as function of total solute molality for HES + NaCl + water solutions,  $R$  values ranging from 0.5 to 20. The symbols are data from Jochem and Korber.<sup>57</sup> The solid lines are the rearranged multisolute OVE, eq 19, fit to the data.

number of data points. Since the SSE depends on the number of data points and each multisolute solution has a different number

**Table 4.** HES Osmotic Virial Coefficients for Varying Mass Ratios ( $R$ ) of HES + NaCl + Water Solutions<sup>a</sup>

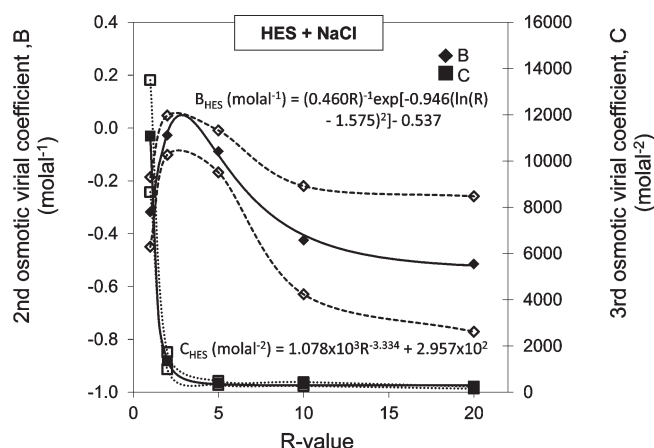
$R = \frac{\text{Mass of HES}}{\text{Mass of NaCl}}$	$B/\text{mol}^{-1}$ [ $\pm 95\%$ CI <sup>†</sup> ]	$C/\text{mol}^{-2}$ [ $\pm 95\%$ CI <sup>†</sup> ]
0.5	$-0.833 [\pm 0.638]$	$7.85 \times 10^4 [\pm 5.68 \times 10^4]$
1.0	$-0.318 [\pm 0.132]$	$1.11 \times 10^4 [\pm 2.42 \times 10^3]$
2.0	$-0.027 [\pm 0.074]$	$1.36 \times 10^3 [\pm 3.71 \times 10^2]$
5.0	$-0.089 [\pm 0.079]$	$3.98 \times 10^2 [\pm 86.8]$
10.0	$-0.425 [\pm 0.205]$	$3.54 \times 10^2 [\pm 83.8]$
20.0	$-0.515 [\pm 0.256]$	$1.96 \times 10^2 [\pm 45.0]$

<sup>a</sup> Data from ref 57.

of data points, the SSE values should only be compared for the different predictions of each multisolute solution and not among the various multisolute solutions.

The percent errors at the maximum solute molality and the SSE for the four predictive multisolute solution theories are listed in Table 3.

The osmotic virial coefficients for the adding-osmolalities approach, the multisolute OVE, and the OVE portion of the PDH–OVE were determined by fitting the single-solute OVE to osmolality as a function of molality data for each of the individual



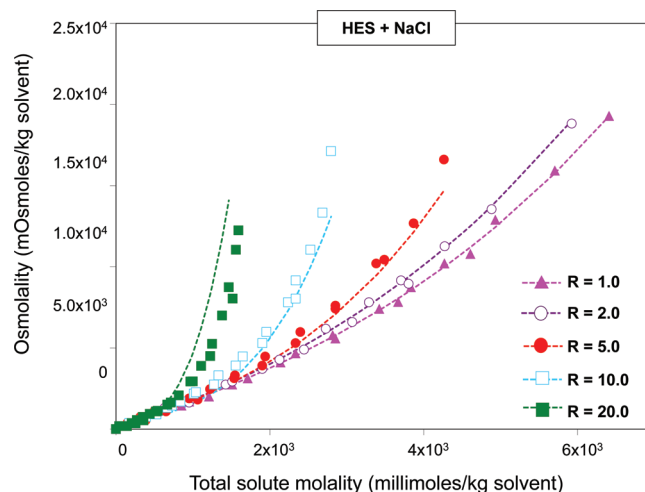
**Figure 7.** Obtained second and third osmotic virial coefficients,  $B$  and  $C$ , for HES in varying concentrations of aqueous salt solutions as a function of  $R$  value. The closed symbols are the values determined for the virial coefficients at each  $R$  value. The solid lines are eq 25 fit to the second virial coefficient ( $B$ ) values as a function of  $R$  value and eq 26 fit to the third virial coefficient ( $C$ ) values as a function of  $R$  value, respectively. The open symbols are the values of the 95% confidence intervals for the osmotic virial coefficients at each  $R$  value. The dashed lines are the upper and lower 95% confidence bands (generated by connecting the values of the upper and lower values of the 95% confidence interval at each  $R$  value with a smoothed line).

solutes (Table 1). The Pitzer–Debye–Huckel parameters for the PDH–OVE are from Pitzer and co-workers<sup>9,51</sup> (Table 2). The results in Table 3 demonstrate that the multisolute OVE and the PDH–OVE methods result in more accurate predictions than the practice of adding osmolalities or assuming an ideal, dilute solution. The only exception is that the practice of adding osmolalities is most accurate for the glycerol + NaCl + water solutions containing high concentrations of glycerol. However, the discrepancy between the measured values and the predictions from the multisolute OVE for solutions containing high concentrations of glycerol may be due to difficulties in accurately measuring the freezing point of highly viscous solutions.

**HES + NaCl + Water Solutions.** The HES + NaCl + water phase diagrams from the literature<sup>57</sup> are shown in Figure 6. The lines on the graph show the fit to each phase diagram to obtain the HES osmotic virial coefficients for each  $R$  value. The HES coefficients obtained by fitting the phase diagrams can be found in Table 4. The values for the HES second and third osmotic virial coefficients,  $B_{\text{HES}}$  and  $C_{\text{HES}}$ , respectively, were graphed as a function of the  $R$  value (Figure 7).

The confidence intervals at varying levels of confidence were determined for each osmotic virial coefficient using a method described previously.<sup>7</sup> The levels of confidence ( $1 - \alpha$ ) were set at a significance level  $\alpha = 0.05, 0.0275$ , and  $0.01$ . For all of the  $R$  values except for the  $R = 0.5$  solution, the confidence interval bands at all levels of confidence followed the same trend as the actual data points. The 95% confidence intervals (i.e.,  $\alpha = 0.05$ ) are shown in Figure 7 and listed in Table 4. The confidence interval bands for the  $C_{\text{HES}}$  values cannot always be clearly seen on the graph because they are very close to the data points.

For the  $R = 0.5$  solution, the confidence interval bands for the osmotic virial coefficients did not follow the same trend as the actual data points at the different levels of confidence. For example, the  $B_{\text{HES}}$  coefficient for the  $R = 0.5$  solution is less than the  $B_{\text{HES}}$  coefficient for the  $R = 1.0$  solution. However, for the



**Figure 8.** Osmolality as a function of total solute molality for HES + NaCl + water solutions,  $R$  values ranging from 1 to 20. The symbols are data from Jochem and Korber.<sup>57</sup> The dashed lines are redrawn from the multisolute OVE, eq 13, using the calculated coefficients from eqs 25 and 26.

95% confidence intervals, the upper confidence limit of the  $B_{\text{HES}}$  value for  $R = 0.5$  was essentially equal to the upper confidence limit of the  $B_{\text{HES}}$  value for  $R = 1.0$ . For the 97.5% and 99% confidence intervals, the upper confidence limit of the  $B_{\text{HES}}$  value for  $R = 0.5$  was greater than the upper confidence limit for the  $R = 1.0$ . Also, at the 99% confidence level, the lower confidence limit for the  $C_{\text{HES}}$  value at  $R = 0.5$  was less than the lower confidence limit for the  $C_{\text{HES}}$  value at  $R = 1.0$ , even though the  $C_{\text{HES}}$  at  $R = 0.5$  solution was greater than the  $C_{\text{HES}}$  for the  $R = 1.0$  solution. The difference between the trends in the data points and in the confidence intervals made it difficult to determine an optimal function to fit to the data when the  $R = 0.5$  coefficient was included.

In addition, the  $R = 0.5$  solution contains twice as much NaCl as HES on a mass basis (or  $1.54 \times 10^4$  times as much on a mole basis). Thus, this solution is most likely dominated by the NaCl solution behavior, making it difficult to obtain accurate HES parameters. For these reasons, the  $R = 0.5$  osmotic virial coefficients were not included in the subsequent analysis to determine a relationship between the osmotic virial coefficients and the  $R$  value.

To provide equations for each of the HES osmotic virial coefficients as a function of  $R$  value, empirical functions of the following forms were fit to the data

$$B_{\text{HES}} (\text{molal}^{-1}) = \frac{1}{aR} \exp[-b(\ln(R) - c)^2] + d \quad (25)$$

$$C_{\text{HES}} (\text{molal}^{-2}) = fR^g + h \quad (26)$$

where  $a = 0.460$ ,  $b = 0.946$ ,  $c = 1.575$ ,  $d = -0.537$ ,  $f = 1.078 \times 10^3$ ,  $g = -3.334$ , and  $h = 2.957 \times 10^2$ .

For aqueous solutions of HES in NaCl, the HES osmotic virial coefficients can be calculated at any  $R$  value using eqs 25 and 26. These equations were used to calculate the osmotic virial coefficients for  $R = 1, 2, 5, 10$ , and  $20$ . Due to the large confidence intervals of the fitted coefficients, the calculated values for the coefficients obtained from eqs 25 and 26 were used to redraw the phase diagrams to determine how well they reproduced the phase diagram data (Figure 8). It can be seen from Figure 8 that

the calculated coefficients accurately redraw the HES + NaCl phase diagram. We also show that the proposed form of the osmotic virial equation can be used to obtain single-solute osmotic virial coefficients of solutes (such as HES) for which the only solution data available are for multisolute solutions containing electrolytes. This is an important illustration since many biologically relevant experiments are performed with the solute of interest dissolved in saline solutions.

## CONCLUSIONS

In the first part of this paper, predictions of the solution behavior for aqueous multisolute solutions containing an electrolyte and a small nonelectrolyte (either DMSO or glycerol) were made using four different predictive multisolute solution theories that do not require fitting of multisolute solution data. Note that regression was used to determine the single-solute parameters but that the multisolute solution osmolalities are predictions. The multisolute solution theories utilized were: (i) ideal, dilute solution theory, (ii) the practice of adding osmolalities, (iii) the multisolute osmotic virial equation (OVE) using the single-solute osmotic virial coefficients and a dissociation constant to capture the electrolyte solution behavior, and (iv) the multisolute OVE using the Pitzer–Debye–Huckel equation<sup>8,9</sup> to capture the electrolyte solution behavior. In both of the multisolute osmotic virial equation approaches, the solution behavior of the nonelectrolyte was captured using the single-solute osmotic virial coefficients, and the interactions between the two different solutes were predicted using a mixing rule for the multisolute OVE proposed by Elliott et al.<sup>28</sup>

For aqueous solutions containing a common CPA (DMSO or glycerol) plus NaCl, it was shown that the predictions from the multisolute OVE using the coefficients from the single-solute osmotic virial equation to capture the electrolyte solution behavior worked as well as using the Pitzer–Debye–Huckel equation to capture the electrolyte solution behavior (Figures 4 and 5, Table 3). However, the OVE approach is much simpler to use, having only one additional fitting constant ( $k_{\text{diss}}$ ) versus the Pitzer–Debye–Huckel function ( $2(f^{\Phi} + 1)$ ), which contains the Debye–Huckel slope ( $A^{\Phi}$ ), an empirical parameter ( $b$ ), and the ionic strength of the solution ( $I$ ). In addition, the osmotic virial coefficients for the electrolyte are constants, whereas in the Pitzer–Debye–Huckel equation the second virial coefficient ( $B_{\text{MX}}^{\Phi}$ ) is also a function of ionic strength and is calculated using three additional empirical constants ( $\beta_{\text{MX}}^{(0)}$ ,  $\beta_{\text{MX}}^{(1)}$ , and  $\alpha$ ). The multisolute OVE was demonstrated to be an accurate predictive solution theory for the multisolute solutions containing one electrolyte considered herein, except in the glycerol + NaCl system at high glycerol concentrations.

In addition, it has been shown that in most cases the multisolute OVE approach is more accurate over a range of concentrations than adding osmolalities or assuming the solutions are ideal and dilute.<sup>7,18,28</sup> Adding osmolalities does provide accurate predictions for some solutions<sup>29</sup> but cannot be used once the solutions become increasingly nonideal.<sup>7,28</sup> This is due to the fact that the adding-osmolalities approach does not take into account the interactions between the different types of solute molecules (i.e., between the electrolyte and the CPA), and these interactions become increasingly important as the concentration increases. The predictions from the multisolute OVE for the glycerol + NaCl + water solutions are not as accurate as the predictions from the adding-osmolalities approach, but this may

be due to difficulties in measuring the freezing point of increasingly viscous glycerol solutions.

In the second part of this paper, the use of the multisolute OVE approach was investigated for aqueous solutions of a macromolecule (HES) plus an electrolyte (NaCl) to determine if the multisolute OVE could be used to capture the changing macromolecular solution behavior in varying concentrations of electrolytes. The aqueous HES + NaCl system was chosen as a model since it plays an important role in biology.<sup>45–48</sup> The multisolute OVE was rearranged so that the single-solute HES osmotic virial coefficients could be obtained from multisolute solution data. Phase diagrams for different mass ratios of HES to NaCl in water ( $R = 0.5, 1, 2, 5, 10$ , and  $20$ ) were obtained from the literature<sup>57</sup> and the coefficients for HES obtained for all of the  $R$  values (Figure 6 and Table 4) by regression. It can be seen that the varying salt concentration plays a role in the HES solution behavior, as there is a marked change in the HES osmotic virial coefficients as the mass ratio of HES to NaCl (the  $R$  value) is changed. The dependence of the HES osmotic virial coefficients on the salt concentration was determined by plotting the HES osmotic virial coefficients as a function of  $R$  value and fitting a function to the data (Figure 7). Empirical equations obtained by fitting the HES osmotic virial coefficient values, eqs 25 and 26, can be used to calculate the osmotic virial coefficients for HES in other concentrations of salt solutions. The calculated coefficients can then be used to redraw the HES + NaCl + water solution behavior (Figure 8). This study demonstrates that the multisolute OVE approach is a simple method to model the effect of salts on macromolecules and that the multisolute OVE approach can be used to back out single-solute osmotic virial coefficients for the macromolecule even when the only solution data available are for the macromolecule in combination with an electrolyte.

Similar work using the virial coefficients to investigate how the solution behavior of a macromolecule is affected by electrolytes has been done on aqueous solutions of lysozyme + NaCl.<sup>39</sup> In that study the virial coefficients were calculated from predictions of the potential mean force between two lysozyme proteins in solutions with varying ionic strength and pH, and the predictions were compared with experimental measurements. A dependence of the second virial coefficient on NaCl concentration was observed. As the NaCl concentration increased from approximately 0.2 molar to 1.75 molar, the second virial coefficient of lysozyme decreased<sup>39</sup> from approximately  $3 \times 10^4$  mL/mol/g<sup>2</sup> to  $-8 \times 10^4$  mL/mol/g<sup>2</sup>. Although the dependence on salt concentration does not follow the same trend as we have observed with HES, there is still a marked dependence on the salt concentration. The differences in the behavior of the macromolecules with changes in salt concentration may be due to differences in the structure and composition of the macromolecules: HES is a starch, and lysozyme is a globular protein. As the experimental data for the lysozyme + NaCl solutions consisted of only one data point for each NaCl concentration, our equation could not be fit to the data to obtain the lysozyme osmotic virial coefficients.

There is other evidence of salts affecting macromolecule solution behavior.<sup>34–37</sup> Unfortunately, in each of these studies it was either not entirely clear how the osmolality and final solution composition were measured, or the osmolality of the solutions was only measured for one concentration of electrolyte. Thus, the same type of analysis as was done with HES + NaCl + water data in this study could not be done on those data sets.



The form of the multisolute OVE recently proposed,<sup>7,28</sup> utilizing mixing rules derived from thermodynamic principles, requires only single-solute information to make predictions of multisolute solution behavior. The application of the OVE to biological solutions is extremely attractive due to the wide range of solutes of interest and the seemingly limitless combinations of these solutes. It has been shown to be accurate for a wide range of multisolute solutions, including those solutions containing two small molecules, a protein and an ideal solute, or two proteins,<sup>7,28</sup> and now for solutions containing electrolytes. In addition, this form of the multisolute OVE can be regressed to provide a practical description of HES–NaCl–water osmolalities.

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