Thermodynamic Expressions Relating Different Types of Preferential Interaction Coefficients in Solutions Containing Two Solute Components

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Received: July 11, 2001; In Final Form: September 20, 2001

In aqueous solutions that contain a macromolecular solute (charged or uncharged) and molecules or ions of a smaller solute, differences between solute−macromolecule and water−macromolecule interactions have solute-concentration-dependent thermodynamic effects that are characterized quantitatively by "preferential interaction" coefficients. Of primary interest in this paper are Γ_{μ_1} and Γ_{μ_3}, defined as partial derivatives that specify the dependence of the molality of the smaller solute on the macromolecular molality at fixed temperature and pressure and the chemical potential indicated by the subscript (μ_1 for water and μ_3 for the smaller solute). Coefficients of the type Γ_{μ_3} (but not Γ_{μ_1}) are direct gauges of thermodynamic effects due to the preferential interactions of a small solute with a macromolecule. Although individual values of Γ_{μ_3} cannot generally be obtained directly by any experimental method, corresponding values of Γ_{μ_1} are directly measurable for involatile solutes ($0.01 \lesssim m_3 \lesssim 3$ *m*) by an accurate, efficient method based on water vapor pressure osmometry [Courtenay et al. *Biochemistry* **2000**, *39*, 4455]. In that study, alternative approximate expressions, for which we present generalized derivations here, were used to calculate Γ_{μ_3}. The resulting values differ significantly from the corresponding values of Γ_{μ_1} for the interactions of various small biochemical solutes with a common globular protein. To identify the general physical origins and thermodynamic implications of numerical differences between Γ_{μ_3} and Γ_{μ_1}, we derive an exact thermodynamic relationship linking these coefficients and examine how it is affected, for various types of systems and ranges of conditions, by contributions from the ideal mixing entropy of components 2 and 3 and from the nonideality due (primarily) to interactions of the macromolecule with the smaller solute. This analysis shows why, in general, Γ_{μ_3} differs significantly from Γ_{μ_1} and why $\Gamma_{\mu_3} \cong \Gamma_{\mu_1}$ in the exceptional cases where components 3 and 2 have a common ion present in large excess over the macromolecular species.

I. Introduction

The interactions of small solute molecules or ions with a macromolecule in solution differ significantly in physical character from the interactions of water molecules with the macromolecular surface. As an important thermodynamic consequence of these "preferential" interactions, the macromolecular chemical potential typically has a strong dependence on the concentration of the small solute. Therefore, a process involving at least one macromolecule as a stoichiometric participant can be driven toward reactants or products by changing the concentration of a "perturbing" solute that interacts with, but does not actually bind to, the macromolecule. Numerous quantitative experimental investigations of protein denaturation, ligand−nucleic acid binding and other biopolymer processes utilize as the perturbing solute a denaturant, osmolyte, or "Hofmeister" salt. The results of such studies and of "osmotic stress" experiments (which utilize changes in the concentration of an osmolyte to perturb processes involving proteins and/or nucleic acids) can be rigorously analyzed and interpreted in terms of thermodynamic functions that characterize the preferential interactions of small solutes with biopolymers.[1−7]

Various partial derivatives commonly (though not invariably) called "preferential interaction coefficients" specify how the concentration of one solute varies with that of another when temperature and the appropriate number of additional thermodynamic functions, including at least one chemical potential, are held constant. Most of the direct experimental determinations, and theoretical calculations, of preferential interaction coefficients have been reported for solutions comprised of water and two types of solute components whose concentrations can be varied independently. According to the prevalent numerical labeling convention, "1" denotes the solvent, "2" denotes a solute component composed either of macromolecules with no net charge or of macroions and an equivalent number of dissociated counterions, and "3" denotes a solute component that consists of net neutral molecules or dissociated oppositely charged ions, which typically are much smaller than the macromolecule and at least somewhat more concentrated.

To facilitate cross reference and comparisons of similar but distinct mathematical forms, Table 1 lists the definitions and alternative representations of four different types of preferential interaction coefficients that are needed for the derivations and discussion presented at various places throughout this paper. Each of these coefficients is a partial derivative that specifies how the molality of component 3 must change as the molality of component 2 is changed to hold constant temperature and two of the following three thermodynamic variables: μ_1, μ_3,

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10.1021/jp012627p CCC: $22.00
Published on Web 12/20/2001

TABLE 1: Definitions and Alternative Representations of Preferential Interaction Coefficients for Solutions Containing Two Solute Components

definitions[a]		alternative representations[b]	
$\Gamma_{\mu_1} \equiv (\partial m_3/\partial m_2)_{T,P,\mu_1}$	(1a)	$\Gamma_{\mu_1} = -\mu_{12}/\mu_{13}$	(1b)
$\Gamma_{\mu_2} \equiv (\partial m_3/\partial m_2)_{T,P,\mu_2}$	(2a)	$\Gamma_{\mu_2} = -\mu_{22}/\mu_{23} = -(\partial\mu_2/\partial\mu_3)_{T,P,m_3}$	(2b)
$\Gamma_{\mu_3} \equiv (\partial m_3/\partial m_2)_{T,P,\mu_3}$	(3a)	$\Gamma_{\mu_3} = -\mu_{32}/\mu_{33} = -(\partial\mu_2/\partial\mu_3)_{T,P,m_2}$	(3b)
$\Gamma_{\mu_1,\mu_3} \equiv (\partial m_3/\partial m_2)_{T,\mu_1,\mu_3}$	(4)		

[a] As usual, m_i represents moles of solute component i (2 or 3) per kg of solvent (1), which conventionally is assumed to consist of all of the water in the solution, including any that may be stoichiometrically bound "as ligand" to the macromolecule or to small solute ions or molecules. Corrected values of solute molalities, defined with respect to the actual amount of "bulk" solvent, can be calculated if the total amount of water bound to the various solute species is known. [b] In accordance with the conventional double-subscript notation, the derivative of the chemical potential of any of the three components with respect to the molality of either solute component is represented by $\mu_{ij} \equiv (\partial\mu_i/\partial m_j)_{T,P,m_k}$, where $i = 1$, 2, or 3, $j = 2$ or 3, and $k \neq j$. By the property of Euler reciprocity, the cross partial derivatives μ_{jk} and μ_{kj} are equal when the subscripts j and k refer to either of the solute components, 2 or 3, but the symbol μ_{k1} is meaningless for $k = 1$, 2, 3. Thus, Euler reciprocity does not apply to derivatives of the type μ_{1k}.

and P. For a "three-component" solution in a given thermodynamic state, the coefficients defined in eqs 1a, 2a, 3a, and 4 are expected, in general, to have different numerical values simply because the different sets of constraints on these partial derivatives cannot under all conditions produce identical functional dependences of m_3 on m_2. This mathematical expectation has been confirmed experimentally for Γ_{μ_1} and Γ_{μ_3}, which have been found to differ significantly in solutions in which the macromolecule is the globular protein BSA (bovine serum albumin) and component 3 is any of a wide variety of biochemical solutes at concentrations in the range $0.01 \lesssim m_3 \lesssim 1$ m. These results were obtained by novel applications of water vapor pressure osmometry (VPO).[2,3,8]

Prior to the development of VPO as a quantitative method of investigating preferential interactions, the relationship between Γ_{μ_1} and Γ_{μ_3} had been considered, to the best of our knowledge, only indirectly (as summarized on pp 45−46 of ref 7) and only for solutions containing high (>1.5 M) concentrations of NaCl and dilute concentrations of the sodium salt of polymeric (1000 bp) DNA. For this system $\Gamma_{\mu_3} \cong \Gamma_{\mu_1}$, as indicated implicitly by comparing each of these coefficients separately with Γ_{μ_1,μ_3}. These comparisons are based on thermodynamic relationships that incorporate certain approximations and experimental input obtained from equilibrium dialysis[9] (for Γ_{μ_1,μ_3}) and isopiestic distillation[10] (for a coefficient closely related to Γ_{μ_1}). On the basis of thermodynamic relationships derived in this paper, we show that the approximate equality of Γ_{μ_1} and Γ_{μ_3} in solutions containing NaCl and NaDNA is an understandable exception to the general expectation of significant differences between Γ_{μ_1} and Γ_{μ_3}, which have been reported[3,8] for solutions in which the macromolecule is a protein and the interacting small solute is any of a diverse group of nonelectrolytes, salts, and zwitterions at concentrations over ranges including physiological conditions.

The inequality of Γ_{μ_3} and Γ_{μ_1} has a substantial impact on comprehensive characterizations of preferential interactions because the coefficient of more direct physical significance, Γ_{μ_3}, cannot generally be determined by any direct experimental method, whereas corresponding values of Γ_{μ_1} can be quantified accurately by analyzing VPO measurements.[2,3] In this paper, we derive a rigorous thermodynamic relationship between Γ_{μ_3} and Γ_{μ_1} and investigate its practical and theoretical implications. For the various classes of three-component solutions specified in the Appendix (first section), we derive alternative expressions for calculations of Γ_{μ_3} with input from the same kinds of VPO measurements that suffice to calculate Γ_{μ_1}. To determine the physical basis for the broad range of applicability of this approach, we examine how the fundamental thermodynamic linkage of Γ_{μ_3} and Γ_{μ_1} is affected by contributions from nonideality and ideal mixing entropy. Expressions for the latter, as explicit functions of concentration variables and the net macromolecular charge, are presented in the Appendix for general classes of three-component solutions, which are distinguished only by whether the constituent solute species are charged and, if so, whether the macromolecular counterion is of the same type as one of the ions dissociated from component 3. On the basis of these expressions, we also identify general characteristics of those exceptional systems in which numerical differences between Γ_{μ_3} and Γ_{μ_1} are expected to be insignificant.

As background essential to clarify the scientific significance of the results presented in this paper, section II-A explains the necessity, for our derivations, of representing preferential interaction coefficients in terms of chemical potential derivatives and discusses some physical implications of these representations; section II− B explains the primary reason coefficients of the type Γ_{μ_3} have a more direct physical significance than those of the type Γ_{μ_1}; section II−C discusses the comparative advantages of VPO as a quantitative method of investigating preferential interactions. The ultimate thermodynamic origin of all of the expressions derived in this paper, the Gibbs−Duhem-based linkages among derivatives of μ_2 and μ_3, are introduced in section III-A and used to derive an exact relationship that links the three coefficients of the type Γ_{μ_k} in any three-component system. This relationship, in a simplified form applicable to solutions in which the macromolecule is sufficiently dilute, is used in section III−B to show how the difference between Γ_{μ_3} and Γ_{μ_1} is determined by the ideal mixing entropy of component 2 and by the nonideality of component 3. In section III−C, we present and discuss generalized derivations of two alternative approximate expressions for Γ_{μ_3} that can be applied to analyze the appropriate VPO measurements on any three-component system. In section III-D, using expressions presented in the Appendix, we examine how contributions from ideal mixing entropy affect the magnitude of Γ_{μ_2} and hence the relationship between Γ_{μ_1} and Γ_{μ_3}. Specifically, we investigate how the relative difference $(\Gamma_{\mu_1} - \Gamma_{\mu_3})/\Gamma_{\mu_1}$ is affected by the dependence of the ideal mixing entropy of the macromolecular component on solution composition and on the net macromolecular charge for the various general classes of three-component systems.

II. Preferential Interaction Coefficients: Thermodynamic Significance and Experimental Accessibility

II-A. Physical Interpretations and Theoretical Applications. *Comparison with Activity Coefficients.* Because activity coefficients generally are more familiar than preferential coefficients, we consider first a qualitative comparison of these different thermodynamic gauges of nonideality in solutions containing at least two solute components. (In this paper, "component" refers to a molecular species with no net charge or an electroneutral combination of two dissociated charged species.) Precise mathematical definitions of the various coefficients that characterize preferential interactions in a three-component solution are given in Table 1, together with alternative representations in terms of derivatives of chemical potentials, which are required to relate coefficients such as Γ_{μ_3} to derivatives involving the activity coefficients of components 2 and 3.

Although several definitions are in common use,[11] the activity coefficient of a solute species or component *i* has the most directly interpretable physical significance when it is defined as the ratio of activity to mole fraction: $\gamma_i \equiv a_i/x_i$. For each chemically distinct species (charged or uncharged) in a homogeneous solution, the sign and magnitude of $\ln \gamma_i$ are determined by the collective thermodynamic consequences of its interactions with all species (including itself) in the system, as compared with a (hypothetical) "ideal dilute" reference solution in which, regardless of its concentration, *i* interacts only with solvent molecules. The sign of $\ln \gamma_i$ is determined by whether the totality of the interactions experienced by species (or component) *i* have a thermodynamically favorable ($\ln \gamma_i < 0$) or unfavorable ($\ln \gamma_i > 0$) effect on its chemical potential in the real solution as compared with the standard state chemical potential of *i* in the reference solution.

Whereas the activity coefficient of each component characterizes only its own nonideality, a preferential interaction coefficient of the type Γ_{μ_3} (or Γ_{μ_2}) expresses the correlation between changes in the chemical potentials of a particular *pair* of components when the concentration of one of them is changed. This correlation is determined not only by the concentration dependences of the nonideality of each component but also by contributions from their ideal mixing entropies. Thus, in eq 3b, the derivative $\mu_{32} = \mu_{23}$ can be resolved into contributions from the nonideality of component 2 (expressed by the "excess" chemical potential, $\mu_2^{ex} \equiv RT \ln \gamma_2$) and from ideal mixing entropy (μ_2^{mix}). Exact expressions for μ_2^{mix} and its derivatives with respect to m_2 and m_3 are presented in the Appendix. This entropic contribution is not always negligible compared with that from nonideality (μ_2^{ex}), in particular for solutions in which components 2 and 3 have a common ion. In general, the concentration dependences of μ_2^{ex}, μ_2^{mix}, μ_3^{mix}, and μ_3^{ex} all must be taken into account for the purpose of assessing the physical origins of significant numerical differences between Γ_{μ_3} and Γ_{μ_1}.

Utility of Alternative Representations. The representations of coefficients of the type Γ_{μ_k} given in Table 1 (by eqs 1b, 2b, or 3b) are needed in this paper for two reasons. First, our derivations of relationships among different types of preferential interaction coefficients proceed from the Gibbs−Duhem equation, which provides rigorous linkages among the derivatives of the chemical potentials of all three components. Second, when Γ_{μ_3} or Γ_{μ_2} is represented as a quotient of derivatives of μ_2 and μ_3, contributions to each of these derivatives from the ideal mixing entropy of components 2 and 3, as given by the expressions presented in the Appendix, can be examined separately from corresponding contributions due to concentration-dependent nonideality. This approach is utilized in section III-C to derive alternative approximate expressions for Γ_{μ_3} and in section III-D to examine the relationship between Γ_{μ_3} and Γ_{μ_1}.

All of the alternative representations of Γ_{μ_k} given in Table 1 follow from various standard mathematical characteristics of partial derivatives. (For example, our eqs 1b and 3b correspond exactly, with appropriate changes in notation, to eqs 2.75 and 2.71, respectively, in Chapter 2 of ref 7.) Each coefficient of the type Γ_{μ_k}, as represented by eq 1b or the first equalities in eqs 2b and 3b, is a quotient of derivatives that compares effects on the *same* chemical potential, μ_k, produced by two *different* changes in state (specifically, changing either m_2 or m_3). In contrast, each of the forms of Γ_{μ_2} and Γ_{μ_3} given by the second equalities in eqs 2b and 3b, respectively, compares effects on two *different* chemical potentials, μ_2 and μ_3, caused by the *same* change in state: varying either m_2 for Γ_{μ_2} or m_3 for Γ_{μ_3}. Consequently, each of these two coefficients is a chemical potential coupling derivative that expresses the functional interdependence of μ_2 and μ_3, subject to the indicated constraints.

Representing Γ_{μ_3} as the partial derivative of one chemical potential with respect to another is crucial for the rigorous demonstration[6,12] that coefficients of this type are directly useful for analyzing effects on a macromolecular process that are caused by changing the concentration of an excess perturbing solute (component 3) at a given *T* and *P*. The fundamental thermodynamic basis for such analyses is summarized below in section II-B. Unlike Γ_{μ_3} and Γ_{μ_2}, the "isoosmolal" preferential interaction coefficient, Γ_{μ_1}, cannot be represented exactly as a (single) chemical potential coupling derivative (for the reason explained in the footnote b to Table 1). Consequently, despite the more direct experimental accessibility of Γ_{μ_1}, it is not as directly useful as Γ_{μ_3} for rigorous analyses and interpretations of thermodynamic and molecular effects due to preferential interactions.

Thermodynamic Interpretation of the Sign of a Chemical Potential Coupling Derivative. In a homogeneous solution containing two or more solute components at constant *T* and *P*, an increase in the molality of (only) one of them must raise its own chemical potential.[13] However, this change in state may either raise or lower (or, in singular cases, leave unchanged) the chemical potential of any other solute component, as long as the total Gibbs free energy of the (properly defined) system is lowered. On the basis of these fundamental considerations, eqs 2b and 3b require that Γ_{μ_2} and Γ_{μ_3} must have the same sign, determined by whether μ_{23} is positive or negative. Thus, an increase in m_3 at fixed m_2 produces a coupling between μ_2 and μ_3 that is thermodynamically favorable when $\Gamma_{\mu_3} > 0$ (because μ_2 is lowered while μ_3 is raised) or unfavorable when $\Gamma_{\mu_3} < 0$ (because both μ_2 and μ_3 are raised). Analogously, the (generally much stronger) coupling between μ_2 and μ_3 produced by an increase in m_2 at fixed m_3 is thermodynamically favorable when $\Gamma_{\mu_2} > 0$ (because μ_3 is lowered while μ_2 is raised) or unfavorable when $\Gamma_{\mu_2} < 0$ (because both μ_2 and μ_3 are raised). Because Γ_{μ_1} cannot be represented as a chemical potential coupling derivative, the sign of this coefficient has a less straightforward thermodynamic interpretation and its correlation with the sign of Γ_{μ_3} (and Γ_{μ_2}) is determined by expressions (derived in section III-A) based on the Gibbs−Duhem equation.

II-B. Role of Chemical Potential Coupling Derivatives in Analyzing Solute-Concentration-Dependent Effects on a Macromolecular Process. In experimental investigations of processes such as protein denaturation or the binding of an oligocationic ligand to a nucleic acid, values of the "observed equilibrium constant", K_{obs}, typically are reported. For a given process, this thermodynamic function is defined, by analogy to the equilibrium constant, as the corresponding stoichiometric quotient of the equilibrium molarities (instead of activities) of the reactant(s) and product(s) at specified *T* and *P*. Without actually binding to any reactant or product, the molecules or ions of component 3 generally still interact, as mobile species in solution, with each stoichiometric participant. Because of differences in the thermodynamic effects of these interactions, the activity of each reactant or product is a different function of a_3. Consequently, a change in a_3 generally shifts the process either toward reactants or toward products.[6,12] When at least one of these is macromolecular, the effect of the perturbing solute on the process typically is large enough so that K_{obs} has a substantial dependence on a_3.

Effects of a perturbing salt on the binding of an oligocationic ligand to a polymeric nucleic acid are characterized experimen-

tally by determining the derivative $SK_{obs} \equiv (\partial \ln K_{obs}/\partial \ln C_3)_{T,P}$. As a consequence (primarily) of the "polyelectrolyte effect", SK_{obs} is negative with a magnitude proportional to the charge on the ligand and relatively independent of C_3.[14,15] In contrast, if the perturbing solute is a denaturant or other osmolyte, $\ln K_{obs}$ is an approximately linear function of C_3 for a process such as the unfolding of a protein induced by changing the concentration of a destabilizing solute (such as urea or a guanidinium salt).[16−20] Accordingly, experimental investigations of protein denaturation report the slope of a plot of $\ln K_{obs}$ vs C_3 (proportional at constant T to the so-called "m-value".[16,17])

Under the typical conditions in which experimental values of K_{obs} are determined (commonly by some spectroscopic method), all solutes other than component 3 are highly dilute and the concentration of this perturbing solute is the only thermodynamic variable that can affect the activity coefficients of the reactants and products at a given T and P. For such systems, the effect of a change in a_3 on the activity of each stoichiometric participant in the process can be represented by a coefficient defined for a solution containing only this reactant or product (component 2) and the perturbing solute (component 3):

$$\Gamma_{\mu_3} = -(\partial \ln a_2/\partial \ln a_3)_{T,P,m_2} \quad (5)$$

This expression follows from the second equality in eq 3b and the conventional definition relating a chemical potential to the corresponding thermodynamic activity and a standard-state chemical potential that depends only on T and P. The functional coupling of activities expressed by eq 5 enters at a fundamental level into thermodynamic analyses[6,12] of processes for which K_{obs} has a substantial dependence on a_3 due to differences in the thermodynamic effects of the preferential interactions of the perturbing solute with the various reactants and products.

For the purpose of introducing preferential interaction coefficients into model-independent analyses of the effects of a perturbing solute on a process, the dependence of K_{obs} on a_3 is expressed as the derivative:

$$S_a K_{obs} \equiv \nu(\partial \ln K_{obs}/\partial \ln a_3)_{T,P} \quad (6)$$

In systems in which the perturbing solute is a molecule with no net charge, $\nu = 1$. If component 3 dissociates completely into cations with charge z_+ and anions with charge z_-, then $\nu = \nu_+ + \nu_-$, where $\nu_+ \equiv |z_-|$ and $\nu_- \equiv z_+$. In typical experimental investigations of solute effects on K_{obs}, the contribution of nonideality to the m_3 dependence of a_3 is determined (to a sufficient approximation) entirely by the interactions of the molecules or ions of component 3 with each other. Hence, experimental values of SK_{obs} or $(\partial \ln K_{obs}/\partial C_3)_{T,P}$, as appropriate for the type of process,[3,6] can be converted to the corresponding derivative of K_{obs} with respect to solute activity by using (for example) VPO measurements on solutions in which the only solute is component 3.

For a given process at constant T and P, $S_a K_{obs}$ is related to the pertinent preferential interaction coefficients by the following model-independent expression:[6]

$$S_a K_{obs} = \Delta|Z| + \nu\Delta\Gamma_{\mu_3} \quad (7)$$

Here the symbol Δ (as in $\Delta G°$) denotes a "stoichiometric combination" of terms, each pertaining to a different reactant or product; for each of these, Z denotes the *net* number of structural charges, which are assumed to be invariant to changes in the concentration of the perturbing solute and to concomitant changes in the concentrations of the reactants and products. These stoichiometric participants need not be macromolecular, but all must be at concentrations sufficiently dilute so that m_3 is the only independent concentration variable upon which K_{obs} depends significantly.

Most of the processes for which K_{obs} has been determined experimentally involve charged species. At the molecular level, none of these participates in the process as an electroneutral "unit". Nevertheless, for each charged reactant or product species, the Γ_{μ_3} that appears in eq 7 is, as required by eq 5, a derivative that expresses the functional coupling of $\ln a_3$ to the logarithm of the activity of the corresponding electroneutral component. Therefore, the combination $\Delta|Z|$ in eq 7 represents a net contribution to $S_a K_{obs}$ from the small ions needed to balance charges on the various reactants and products. Although $\Delta|Z| = 0$ for most processes involving like-charged species (such as conformational transitions of nucleic acids without coupled changes in their extents of protonation), generally $\Delta|Z|$ is not small compared with $\nu\Delta\Gamma_{\mu_3}$ for processes that involve oppositely charged reactants (such as the binding of an oligocationic ligand to a nucleic acid).[4,6]

In the rigorous derivation[6] of eq 7, no assumptions are made concerning the m_3 dependence of any of the Γ_{μ_3} that characterize the preferential interactions of the perturbing solute with the various reactants and products of the process for which K_{obs} is defined. Consequently, in solutions in which all of the stoichiometric participants are sufficiently dilute compared with the perturbing solute, eq 7 constitutes the most fundamental, *model-independent* way of expressing the dependence of K_{obs} on a_3. With this equation, experimental values of $S_a K_{obs}$ can be related to values of Γ_{μ_3} determined independently for solutions that consist only of solvent, the perturbing solute component ("3"), and a component ("2") corresponding to one of the stoichiometric participants. Such determinations can be based on various types of theoretical calculations[21−26] or on information obtained by analyzing the results of various experimental measurements, summarized in the next section. For any protein process perturbed by changing the concentration of a solute (either a denaturant or an osmolyte), the sign and magnitude of $(\partial \ln K_{obs}/\partial C_3)_{T,P}$ can be interpreted with the "local-bulk domain" model for solute−biopolymer preferential interactions.[1,4,27]

II-C. Experimental Methods of Quantifying Preferential Interaction Coefficients. Under the typical experimental conditions in studies of the effects of an excess perturbing solute on a macromolecular process, eq 7 provides the rigorous thermodynamic relationship whereby these effects can be analyzed in terms of preferential interaction coefficients. Although $S_a K_{obs}$ is experimentally accessible, individual values of the coefficients comprising $\Delta\Gamma_{\mu_3}$ generally cannot be determined directly by any method.

In practice, the constraints on the partial derivative that defines Γ_{μ_3} according to eq 3a generally cannot be imposed by equilibrating simultaneously a series of solutions that contain various amounts of components 2 and 3 with one solution in which the only solute is component 3.[28] If the solubility of component 3 could be saturated in a series of solutions containing various amounts of components 2 and 3 (without precipitation of component 2), the constancy of μ_3 in all of these systems would be maintained at a given T and P and Γ_{μ_3} could be determined as the slope of a plot of m_3 vs m_2. However, this information generally would not suffice for rigorous analyses and interpretations of effects due to preferential interactions under the conditions at which they typically are investigated.

For practical and accurate quantitative determinations of Γ_{μ_3} as well as Γ_{μ_1}, Zhang et al.[2] and Courtenay et al.[3] developed

methods based on input accessible by VPO. These measurements can be made with a commercially available water vapor pressure osmometer (such as the Wescor 5500) on solutions in which $m_2 \gtrsim 1$ m*m* and $m_3 \lesssim 3$ *m*. The quantity directly measured is the "osmolality" of the solution, a thermodynamic function related to the solvent activity by $\mathrm{Osm} \equiv -m_1^\bullet \ln a_1$ (where the "water molality" $m_1^\bullet$ has the numerical value of 55.5 mol/kg). In a three-component solution, each of the two independent derivatives of Osm with respect to solute molality is related to the corresponding derivative, required for the Gibbs−Duhem-based analysis presented in section III-A, of the chemical potential of solvent water:

$$(\partial \mathrm{Osm}/\partial m_k)_{T,P,m_j} = -m_1^\bullet \mu_{1k}/(RT) \qquad (8)$$

Here, *k* and *j* refer to either of the solute components 2 and 3. Osmometric determinations of values of μ_{12} and μ_{13} (accurately "matched" so that each pertains to the same state of the system) determine exactly Γ_{μ_1}, as represented by eq 1b. Individual values of these derivatives are needed, in conjunction with VPO measurements on corresponding two-component solutions, as input to the alternative expressions for Γ_{μ_3} derived in section III-C of this paper.

Prior to the development of VPO for experimental determinations of Γ_{μ_1}, the only method available (at least in principle) was isopiestic distillation (ID).[29] This approach, originally developed and extensively applied to measure osmotic coefficients,[30,31] apparently has been utilized in only one experimental study[10] of a biopolymer system to evaluate a coefficient that (as shown by Cohen and Eisenberg[32]) is equivalent to Γ_{μ_1}. As compared with VPO, ID is operationally much more cumbersome and time-consuming (to ensure complete equilibration of a sufficiently large number of solutions with diverse compositions), especially at temperatures and solute concentrations in the ranges (including typical physiological conditions) at which preferential interactions commonly are studied. Separate, *matched* values of μ_{12} and μ_{13} can be determined by ID only by interpolations on a sufficiently large database covering variations in both m_2 and m_3 at various fixed μ_1. According to eq 1b, Γ_{μ_1} determines only the quotient $-\mu_{12}/\mu_{13}$, but individual values of these derivatives are required as input to the equations derived in this paper for the purpose of quantifying Γ_{μ_3} in the wide variety of systems in which this coefficient differs significantly from Γ_{μ_1}.

Most experimental investigations of preferential interactions have used equilibrium dialysis to obtain values of Γ_{μ_1,μ_3}, which are assumed to differ negligibly from the corresponding values of Γ_{μ_3}. In a subsequent paper, we (C. F. Anderson et al.) will examine the general thermodynamic factors that determine when $\Gamma_{\mu_3} \cong \Gamma_{\mu_1,\mu_3}$ on the basis of a rigorous thermodynamic relationship linking these coefficients. Equilibrium dialysis generally is applicable to any solution in which component 2 is too large to diffuse through a membrane that is permeable to solvent water and to the molecules or ions of component 3. When two solutions separated by such a membrane are at dialysis equilibrium, the chemical potential of each diffusible component (1 and 3) is the same, at a fixed temperature, in both solutions. Therefore, as defined by eq 4, Γ_{μ_1,μ_3} can, at least in principle, be quantified as the slope of a plot of m_3 versus m_2 after these molalities have been measured in a series of three-component solutions equilibrated simultaneously with a dialyzing solution containing only components 1 and 3. In current practice, values of Γ_{μ_1,μ_3} usually are obtained less directly, but with substantially greater accuracy, by matching the results of density measurements on a series of simultaneously dialyzed solutions with corresponding results obtained on a series of undialyzed solutions at constant (atmospheric) pressure.[5,33]

Methods based on equilibrium dialysis have been successfully applied to study solute−macromolecule preferential interactions in a wide variety of systems, but this approach is not without operational challenges.[33] The VPO measurements needed to quantify preferential interaction coefficients are made at constant pressure on closed systems, the compositions of which can be determined with high accuracy a priori, when the sample solutions are first prepared. Larger experimental uncertainties are incurred almost inevitably when solute concentrations are determined retroactively, after solutions have been equilibrated by dialysis (or ID). The Appendix of ref 3 provides additional comments about the comparative advantages of VPO for quantitative investigations of solute−macromolecule preferential interactions.

Compared with any method based on dialysis, VPO can be used to quantify preferential interaction coefficients in a greater variety of three-component solutions, provided that components 2 and 3 both are (sufficiently) involatile. For example, the VPO measurements needed to calculate Γ_{μ_3} can be made on solutions in which components 2 and 3 have similar molar volumes (so that both, or neither, would diffuse across a dialysis membrane) or in which two binary solute components dissociate into a total of four charged species that are pairwise electroneutral (as explained in the first section of the Appendix). Such solutions, if dialyzed, would contain a total of *three* independent solute components (unless the macromolecular counterions are too large to be diffusible); therefore, at dialysis equilibrium, the preferential interactions of each of the two small solute components with the macromolecular component could not, in general, be characterized by any of the coefficients in Table 1. For solutions containing one macromolecular and two small solute components, rigorous relationships among preferential interaction coefficients will be derived and utilized subsequently (C. F. Anderson et al., in preparation).

III. Results and Discussion

III-A. Thermodynamic Correlation of Preferential Interaction Coefficients Based on the Gibbs−Duhem Equation. *Gibbs−Duhem Linkages of Chemical Potential Derivatives.* For aqueous solutions containing only one solute component, the Gibbs−Duhem equation provides a rigorous and exceedingly useful thermodynamic relationship between readily measurable changes in the chemical potential of water and corresponding changes in the chemical potential of the solute, which generally are much more difficult (in many cases practically impossible) to measure directly. In such two-component solutions, a change in solute molality at constant temperature and pressure produces changes in the solute and solvent chemical potentials that are related by the Gibbs−Duhem linkage between the derivatives of these components:

$$-m_1^\bullet \mu_{12} = m_2 \mu_{22} \qquad (9)$$

The superscript on $m_1^\bullet$ is used to emphasize that the "molality" of solvent water is fixed. (In some formulations of preferential interaction coefficients, the symbol m_1 denotes a variable equal, or proportional, to the mole ratio, n_1/n_3.) In any aqueous solution in which the single solute component (2) is involatile, VPO measurements can be analyzed using eq 8 to quantify μ_{12} and hence μ_{22} via eq 9.

For solutions that contain two solute components (2 and 3), the two Gibbs−Duhem linkages analogous to eq 9 are as follows:

$$-m_1^{\bullet}\mu_{12} = m_2\mu_{22} + m_3\mu_{32} \tag{10}$$

$$-m_1^{\bullet}\mu_{13} = m_2\mu_{23} + m_3\mu_{33} \tag{11}$$

If both solute components are involatile, matched values of $m_1^{\bullet}\mu_{12}$ and $m_1^{\bullet}\mu_{13}$ (both for the same m_2 and m_3) can be obtained by analyzing the appropriate VPO measurements with equations analogous to eq 8. Osmometric determinations of these two independent derivatives of μ_1 in a three-component solution do not suffice for separate evaluations of the derivatives μ_{22}, μ_{33}, and $\mu_{23} = \mu_{32}$, which are needed to quantify Γ_{μ_3} (or Γ_{μ_2}) as represented by eq 3b (or eq 2b). Nevertheless, in section III-C, we demonstrate that by approximating either μ_{22} or μ_{33}, on the basis of VPO measurements for corresponding two-component solutions, expressions derived from eqs 10 and 11 can be used for independent calculations of Γ_{μ_3}. Thus, this coefficient can be quantified by VPO for systems and conditions in which it differs significantly from Γ_{μ_1}. Physical origins of these differences can be examined on the basis of the relationship linking Γ_{μ_3} to Γ_{μ_2} and Γ_{μ_1} that is derived in the next section.

Gibbs−Duhem Linkage of Preferential Interaction Coefficients. To derive the exact model-independent relationship linking all three coefficients of the type Γ_{μ_k}, eq 10 is divided by eq 11 and eq 1b is used to replace the quotient of derivatives of μ_1 on the left-hand side of the resulting expression by $-\Gamma_{\mu_1}$. On the right-hand side, the numerator and denominator both are divided by μ_{33}, and eqs 2b and 3b are used to introduce the coefficients Γ_{μ_2} and Γ_{μ_3}, respectively. Rearrangement then produces the following expression relating Γ_{μ_3} to Γ_{μ_2} and Γ_{μ_1}:

$$\Gamma_{\mu_3} = \Gamma_{\mu_1}/[1 - (m_2/m_3)(\Gamma_{\mu_2} - \Gamma_{\mu_1})] \tag{12}$$

This purely thermodynamic correlation among preferential interaction coefficients depends explicitly on factors of m_2 and m_3 that originate in the Gibbs−Duhem linkages, eqs 10 and 11. Moreover, as required by the Gibbs Phase Rule for a homogeneous three-component solution, each of the coefficients in eq 12 is, in general, a function of two concentration variables at a given T and P. Neither eq 12 nor any related expression elsewhere in this paper presupposes any specific functional form for the dependence of any preferential interaction coefficient on either m_2 or m_3. The concentration dependence of each type of coefficient is determined ultimately at the molecular level by contributions from ideal mixing entropy, dependent only on the numbers and kinds of chemically distinct species, and from the nonideality due to system-specific intermolecular interaction potentials that depend (with varying degrees of sensitivity) on structural or chemical characteristics of the constituent species.

Equation 12, which can be rearranged in other instructive ways, relates Γ_{μ_3}, the coefficient of primary physical interest (for the reason explained in section II-B), to the following thermodynamic variables: m_2/m_3, the relative amounts of components 2 and 3; Γ_{μ_1}, the isoosmolal coefficient that can be accurately measured by VPO (or ID); and Γ_{μ_2}, a third type of coefficient that, like Γ_{μ_3}, cannot be measured directly under typical conditions of interest. In a subsequent paper, we (C. F. Anderson et al., in preparation) will show that Γ_{μ_2} can be used in rigorous thermodynamic analyses of the effects of solute−macromolecule preferential interactions on macromolecular solubility. In the remainder of this paper, Γ_{μ_2} in eq 12 is the crucial basis for our demonstration that in general Γ_{μ_3} differs significantly from Γ_{μ_1} and for our identification of the special characteristics of systems and conditions for which $\Gamma_{\mu_3} \cong \Gamma_{\mu_1}$. Physical origins and thermodynamic implications of significant differences between Γ_{μ_3} and Γ_{μ_1} are examined in the next section for systems in which m_2 is at high dilution and in section III-D for the more general conditions under which solute−macromolecule preferential interactions are studied by VPO.

III-B. The Relationship between Γ_{μ_3} and Γ_{μ_1} at High Macromolecular Dilutions. *Common Ion Effect on the $|Z_M|$ Dependence of $\Gamma^{\circ(2)}_{\mu_3} - \Gamma^{\circ(2)}_{\mu_1}$.* When component 2 is sufficiently dilute, the term $(m_2\Gamma_{\mu_1}/m_3)$ in the general expression for Γ_{μ_3} given by eq 12 becomes negligible and this equation can be rearranged to the following simpler form, where, on the right-hand side, eq 2b has been introduced to replace Γ_{μ_2} and eq 3b to replace Γ_{μ_3}:

$$\Gamma_{\mu_3} - \Gamma_{\mu_1} \cong m_2\mu_{22}/(m_3\mu_{33}) \tag{13}$$

This expression can be simplified further when the macromolecule is so dilute that the limit $m_2 \to 0$ is applicable. In this limit, the magnitude of $m_2\mu_{22}$ is determined entirely by the contribution from the ideal mixing entropy of the macromolecular component, because μ_2^{mix} diverges as a logarithmic function of m_2. For any of the general classes of three-component solutions specified in the Appendix *except* those in which components 2 and 3 have a common ion, the explicit expression for μ_{22}^{mix} given by eq A-3a is appropriate to determine the limiting value of $m_2\mu_{22}$, and hence of eq 13:

$$\Gamma^{\circ(2)}_{\mu_3} - \Gamma^{\circ(2)}_{\mu_1} = (|Z_M| + 1)RT/(m_3\mu^{\circ(2)}_{33}) \tag{13a}$$

The superscript $^{\circ(2)}$ denotes a thermodynamic function evaluated in the limit $m_2 \to 0$, while T, P, and m_3 (>0) are held fixed. In eq 13a and everywhere else in this paper, the *net* structural charge (if any) on the macromolecule, $|Z_M|$, assumed to consist of univalent groups, does not vary with m_2 or m_3. For simplicity (as explained in the Appendix), even though the macromolecule may have both cationic and anionic structural charges, the electroneutral component 2 is assumed to be "binary", comprised of one macromolecule and a sufficient number of one type of counterion to balance exactly the net macromolecular charge.

In eq 13a, the factor $(|Z_M| + 1)$ originates from the ideal mixing entropy of the macromolecular component, for which μ_{22}^{mix} is determined when m_2 is low enough by $(|Z_M| + 1)/m_2$, as shown by eq A-3a. The divergence of this term in the limit $m_2 \to 0$ is canceled by the factor of m_2 that originates in the Gibbs−Duhem-based eq 10. The corresponding contribution from macromolecular nonideality, μ_{22}^{ex}, must vanish in the limit $m_2 \to 0$ because the interaction between (finite) macromolecules must become negligible as their separation becomes infinite. The factor $m_3\mu^{\circ(2)}_{33}$ in eq 13a consists of a contribution from ideal mixing entropy, $m_3\mu_{33}^{mix}$, as given by eq A-6a in the limit $m_2 \to 0$ and a smaller solute-specific contribution from nonideality, $m_3(\mu_{33}^{ex})^{\circ(2)}$, the numerical significance of which is illustrated in the next section for various biologically prevalent small solutes. Regardless of the chemical nature of component 3, eq 13a shows that the difference between $\Gamma^{\circ(2)}_{\mu_3}$ and $\Gamma^{\circ(2)}_{\mu_1}$ is smallest for an uncharged macromolecule and becomes progressively larger with increasing $|Z_M|$. This $|Z_M|$ dependence arises entirely from the ideal mixing entropy of the dissociated macromolecular counterions.

If $|Z_M| = 0$, eq 13a is applicable to solutions containing any type of small solute species, but if $|Z_M| \neq 0$, this equation applies

only to three-component solutions in which components 2 and 3 do not have a common ion. Otherwise, in solutions containing three charged solute species (one being the common ion), a different simplified form of eq 13 results from applying the limit $m_2 \rightarrow 0$ to μ_{22}^{mix}, as given by eq A-3b:

$$\Gamma_{\mu_3}^{\circ(2)} - \Gamma_{\mu_1}^{\circ(2)} = RT/(m_3\mu_{33}^{\circ(2)}) \qquad (13b)$$

Here, the contribution to $\mu_{33}^{\circ(2)}$ from the ideal mixing entropy of component 3 is given by eq A-6b. Comparison of eq 13b with eq 13a demonstrates that the presence of a common ion reduces $\Gamma_{\mu_3}^{\circ(2)} - \Gamma_{\mu_1}^{\circ(2)}$ by a factor of $(|Z_M| + 1)$ to the value characteristic of a solution in which the macromolecule has no net charge, regardless of the actual magnitude of $|Z_M|$. The physical origin of this effect, clearly substantial for any highly charged macromolecule, can be traced to the reduction in the m_2 dependence of the ideal mixing entropy of component 2 that results when its counterions are commingled with ions of the same type dissociated from component 3.

The derivative $\mu_{33}^{\circ(2)}$ must be positive (for the fundamental reason noted in section II-A). Thus, for systems and conditions in which $\Gamma_{\mu_3}^{\circ(2)} < 0$, eqs 10 and 11 require that $\Gamma_{\mu_1}^{\circ(2)} < 0$. In both eq 13a and eq 13b, the right-hand side must be positive. Therefore, the magnitude of $\Gamma_{\mu_1}^{\circ(2)}$ must exceed that of $\Gamma_{\mu_3}^{\circ(2)}$ when both of these coefficients are negative, and when both are positive $\Gamma_{\mu_3}^{\circ(2)}$ must exceed $\Gamma_{\mu_1}^{\circ(2)}$. However, for systems and conditions in which $\Gamma_{\mu_3}^{\circ(2)} > 0$, the sign of $\Gamma_{\mu_1}^{\circ(2)}$ is not uniquely determined. In accordance with eqs 10 and 11, if $\Gamma_{\mu_3}^{\circ(2)}$ is positive, $\Gamma_{\mu_1}^{\circ(2)}$ can be either positive, negative, or zero, depending on m_3 and on characteristics of the m_3 dependence of $\Gamma_{\mu_3}^{\circ(2)}$. This lack of a strict correlation between the signs of $\Gamma_{\mu_3}^{\circ(2)}$ and $\Gamma_{\mu_1}^{\circ(2)}$ when the former is positive arises because $\Gamma_{\mu_1}^{\circ(2)}$ cannot be represented simply as a chemical potential coupling derivative (as explained in section II-A).

To conclude this general consideration of the common ion effect on the $|Z_M|$ dependence of $\Gamma_{\mu_3}^{\circ(2)} - \Gamma_{\mu_1}^{\circ(2)}$, the limit $m_3 \rightarrow 0$ is applied to eqs 13a and 13b. Theoretical information about this experimentally inaccessible limit is useful, for example, in analyzing VPO measurements to determine preferential interaction coefficients.[2,3] Regardless of the chemical nature of component 3, in all classes of three-component solutions except those in which a common ion is present, $\Gamma_{\mu_3}^{\circ(2)} \rightarrow 0$ as $m_3 \rightarrow 0$ (according to eqs 3b, A-4a, and A-6a); therefore, in this limit, eq 13a shows that $\Gamma_{\mu_1}^{\circ(2)} \rightarrow -(|Z_M| + 1)/\nu$. In contrast, for solutions in which components 2 and 3 have a common ion, according to eqs A-4b and A-6b $\Gamma_{\mu_3}^{\circ(2)} \rightarrow -|Z_M|/\nu$ as $m_3 \rightarrow 0$, and hence, according to eq 13b, $\Gamma_{\mu_1}^{\circ(2)} \rightarrow -(|Z_M| + 1)/\nu$. Thus, when the small solute component is at sufficiently high dilution, the presence of a common ion has a significant, purely entropic effect on the $|Z_M|$ dependence of $\Gamma_{\mu_3}^{\circ(2)}$ but no effect whatever on the $|Z_M|$ dependence of $\Gamma_{\mu_1}^{\circ(2)}$.

In summary, the difference between $\Gamma_{\mu_3}^{\circ(2)}$ and $\Gamma_{\mu_1}^{\circ(2)}$ is greatly reduced by the common ion effect on μ_{22}. A specific example of this reduction is given, on the basis of experimental input for different 1:1 salts, in the following section. Additional examples of substantial common ion effects on the chemical potential derivatives used to represent preferential interaction coefficients, and hence on relationships among them, are discussed in sections III-C and III-D and in the concluding section of the Appendix.

Dependence of $\Gamma_{\mu_3}^{\circ(2)} - \Gamma_{\mu_1}^{\circ(2)}$ *on* m_3 *for Various Common Biochemical Solutes.* Equations 13a and 13b show that the magnitude and solute-concentration dependence of $(\Gamma_{\mu_3}^{\circ(2)} - \Gamma_{\mu_1}^{\circ(2)})$ are determined by the limiting value of the quotient of $m_2\mu_{22}^{\mathrm{mix}}$ and $m_3\mu_{33}^{\circ(2)} = m_3(\mu_{33}^{\mathrm{mix}})^{\circ(2)} + m_3(\mu_{33}^{\mathrm{ex}})^{\circ(2)}$. Thus, at sufficiently high macromolecular dilutions, the difference between $\Gamma_{\mu_3}^{\circ(2)}$ and $\Gamma_{\mu_1}^{\circ(2)}$ depends on the ideal mixing entropy of the macromolecular component but not on any specific characteristics of the macromolecule or its interactions with the small solute component. The derivative $(\mu_{33}^{\mathrm{mix}})^{\circ(2)}$ is given by eq A-6a for all classes of three-component solutions except those in which components 2 and 3 have a common ion, for which $(\mu_{33}^{\mathrm{mix}})^{\circ(2)}$ is given by eq A-6b. The contribution from nonideality, $(\mu_{33}^{\mathrm{ex}})^{\circ(2)}$, is determined by interactions among the species comprising component 3 (relative to their interactions with water) in solutions in which this component is the only solute.

The plots shown in Figure 1 illustrate the magnitude of the inequality of $\Gamma_{\mu_3}^{\circ(2)}$ and $\Gamma_{\mu_1}^{\circ(2)}$ and the m_3 dependence of $(\Gamma_{\mu_3}^{\circ(2)} - \Gamma_{\mu_1}^{\circ(2)})$ for a variety of solutes of which the interactions with proteins have been investigated. Specifically, these include the following: common 1:1 electrolytes (KCl, potassium glutamate, and NaCl), protein destabilizers (urea, guanidinium chloride (GuHCl), and guanidinium thiocyanate (GuHSCN)), and natural osmolytes that stabilize folded states or quaternary assemblies of proteins (glycerol, proline, trehalose, TMAO, and glycine betaine). In accordance with the terminology of the local-bulk domain model,[27] small "perturbing" solutes that interact with a macromolecule can be classified as either "excluded" (if $\Gamma_{\mu_3}^{\circ(2)} < 0$) or "accumulated" (if $\Gamma_{\mu_3}^{\circ(2)} > 0$).

Specific characteristics of the macromolecule, which typically have profound effects on the magnitudes of the individual coefficients $\Gamma_{\mu_3}^{\circ(2)}$ and $\Gamma_{\mu_1}^{\circ(2)}$, nevertheless have no effect on their difference $(\Gamma_{\mu_3}^{\circ(2)} - \Gamma_{\mu_1}^{\circ(2)})$, as given by either eq 13a or eq 13b. Therefore, plots such as those presented in Figure 1 can be constructed without specifying anything about the binary macromolecular component 2 other than the number and type of counterions (if any). Here, we have made the arbitrary choice of $|Z_M| = 6$, a value within the range characteristic of proteins under physiological conditions that moreover allows for a visually informative set of plots for the diverse variety of solutes represented in Figure 1. Because Na^+ is specified as the macromolecular counterion, the contrast between $|\Gamma_{\mu_3}^{\circ(2)} - \Gamma_{\mu_1}^{\circ(2)}|$ for KCl and NaCl exhibits the magnitude of the common ion effect on $m_2\mu_{22}^{\mathrm{mix}}$. The plots shown in Figure 1 were constructed with values of $m_3\mu_{33}^{\circ(2)}$ calculated by using eq 8 to analyze osmometric measurements made in this laboratory, over concentration ranges extending from approximately 0.01 to 1 *m* for each of the eleven solutes. Within experimental uncertainties, our results agree with available corresponding published values of osmotic coefficients, specifically those determined using isopiestic distillation for glycerol (0.1−3.5 *m*),[30] KCl (0.1−1 *m*),[30] NaCl (0.1−0.8 *m*),[30] urea (0.1−2.5 *m*),[30] GuHCl (0.75−1.5 *m*)[31] and KGlu.[34]

The vertical scale of the plots shown in Figure 1 is determined by the macromolecular charge because, in accordance with eq 13a, $(|Z_M| + 1)$ scales the magnitude of $(\Gamma_{\mu_3}^{\circ(2)} - \Gamma_{\mu_1}^{\circ(2)})$ for all systems in which component 3 does not have an ion of the same type as the macromolecular counterion. Although the common ion effect is substantially larger, a significant entropic effect on $(\Gamma_{\mu_3}^{\circ(2)} - \Gamma_{\mu_1}^{\circ(2)})$ also arises from its reciprocal dependence on $m_3\mu_{33}^{\mathrm{mix}}$ in both eqs 13a and 13b. The dissociation of the 1:1 salts reduces $(\Gamma_{\mu_3}^{\circ(2)} - \Gamma_{\mu_1}^{\circ(2)})$ by a factor of 2, as compared with a corresponding solution in which component 3 is any uncharged solute at low m_3. For the wide diversity of solutes represented

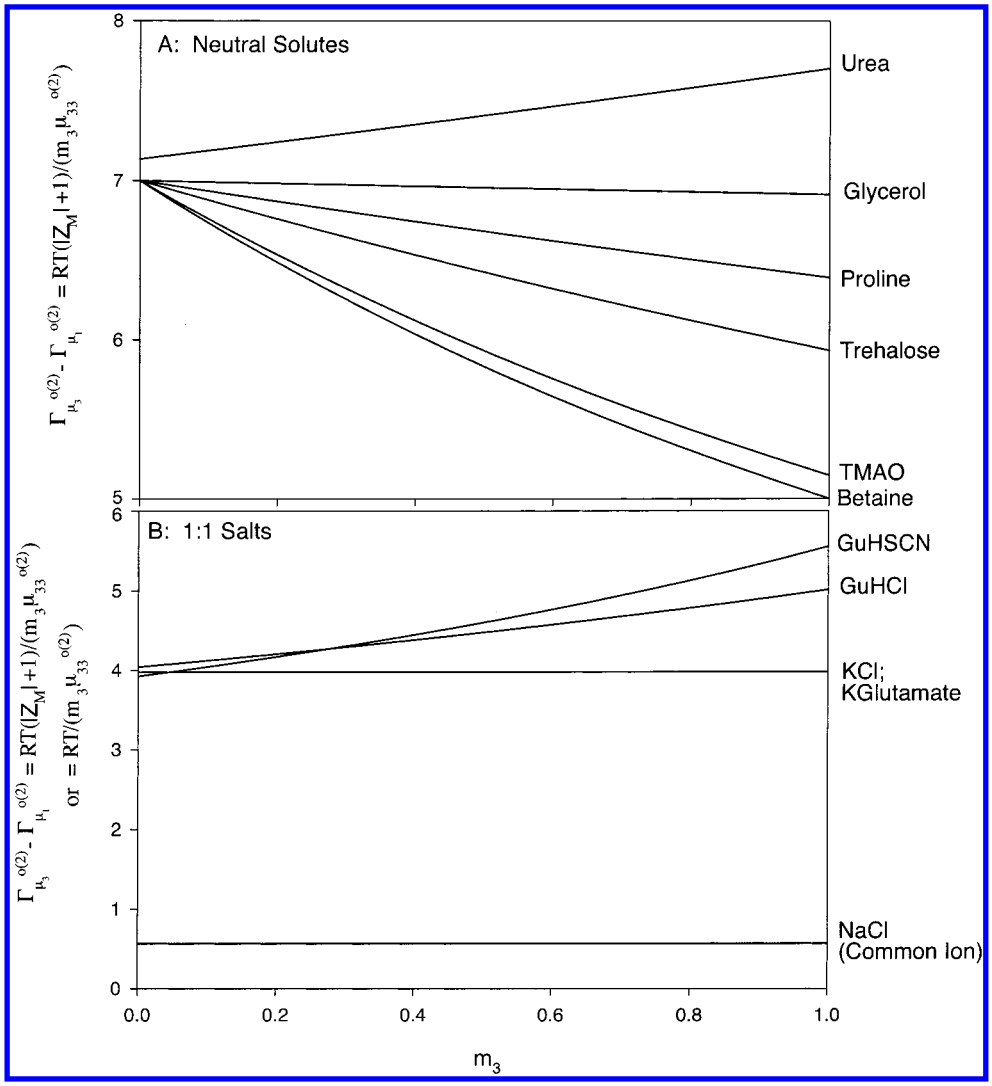

Figure 1. Difference between Γ_{μ_3} and Γ_{μ_1} in the limit $m_2 \rightarrow 0$. The quantity $\Gamma_{\mu_3}^{o(2)} - \Gamma_{\mu_1}^{o(2)}$ is plotted vs solute molality for a hypothetical protein with $Z_M = -6$ and various solutes studied by VPO.[3,8] $m_3\mu_{33}^{o(2)}$ was calculated from solution osmolality as a function of small solute molality in the absence of a macromolecular component using the Gibbs−Duhem relationship analogous to eq 20: $m_3\mu_{33}^{o(2)} = -m_1^{\bullet}\mu_{13}^{o(2)} = RT(\partial\mathrm{Osm}/\partial m_3)_{T,P,m_2=0}$. For these solutes, the average uncertainty in $(\partial\mathrm{Osm}/\partial m_3)_{T,P,m_2=0}$ is 5% and ranges from 2% (for glycerol) to 14% (for urea).

in Figure 1, solute-specific differences in $(\mu_{33}^{ex})^{o(2)}$ have effects on the magnitude of $\Gamma_{\mu_3}^{o(2)} - \Gamma_{\mu_1}^{o(2)}$ that are relatively minor, as compared with the contribution due to the ideal mixing entropy of the macromolecular component (except when component 3 is NaCl because the macromolecular counterion is Na^+). Contributions from $(\mu_{33}^{ex})^{o(2)}$ cause the m_3 dependence of $\Gamma_{\mu_3}^{o(2)} - \Gamma_{\mu_1}^{o(2)}$ to increase as a function of m_3 for the accumulated solutes (urea, GuHCl, and GuHSCN), to decrease for the excluded solutes with no net charge (glycerol, proline, trehalose, TMAO, and glycine betaine), and to remain unchanged for the salts KCl, KGlu, and NaCl. Remarkably, for each of the latter three physiological strong electrolytes, the mean ionic activity coefficient is approximately proportional to some power of m_3 over the experimental range extending from 0.01 to 1.0 *m*.

In summary, comparison of eqs 13a and 13b with eq 12 shows that when the macromolecular concentration is sufficiently dilute, contributions from ideal mixing entropy either completely or largely cancel the factors of m_2 and m_3 that originate in the Gibbs−Duhem linkages given by eqs 10 and 11. The magnitude of $\Gamma_{\mu_3}^{o(2)} - \Gamma_{\mu_1}^{o(2)}$ does not reflect any specific structural or chemical features of the macromolecule or any characteristics of its interactions with the smaller solute species comprising component 3 because in eq 12 the factors of μ_{23} that appear in the representations of Γ_{μ_2} and Γ_{μ_3} given by eqs 2b and 3b cancel when the term $m_2\Gamma_{\mu_1}$ is negligible compared with $m_2\Gamma_{\mu_2}$. In general, however, when the macromolecular concentration is not highly dilute, the magnitude of $(\Gamma_{\mu_3} - \Gamma_{\mu_1})$ is determined not only by the predominantly entropic effects on $\Gamma_{\mu_3}^{o(2)} - \Gamma_{\mu_1}^{o(2)}$ considered in this section but also by effects on the magnitude of μ_{23} that arise from interactions of the small solute molecules or ions with the macromolecule and also, in general, from ideal mixing entropy, especially in solutions in which components 2 and 3 have a common ion. These effects on the relative difference $(\Gamma_{\mu_1} - \Gamma_{\mu_3})/\Gamma_{\mu_1}$ are considered in section III-D for conditions of greater generality than those to which eqs 13a and 13b apply. Toward this objective, equations derived in the following section are needed to calculate values of $(\Gamma_{\mu_1} - \Gamma_{\mu_3})/\Gamma_{\mu_1}$ using VPO measurements.

III-C. Alternative Approximate Expressions for Γ_{μ_3} Based on Gibbs−Duhem Linkages for Two- and Three-Component Solutions. In aqueous solutions containing two involatile solute components, the "isoosmolal" method developed by Courtenay et al.[3] can be applied to determine Γ_{μ_1} at any solute concentration at which the osmolality of the solution falls within the operating range of a vapor pressure osmometer. For the purpose of calculating Γ_{μ_3} by analyzing the same VPO input that can be

used to calculate Γ_{μ_1}, in this section, we derive two expressions, each based on a different approximation. The resulting expressions are applicable to any of the different general classes of three-component solutions specified in the Appendix, although certain terms are significant only in solutions in which components 2 and 3 have a common ion. These calculations require as input, in addition to VPO measurements on three-component solutions, estimates of either μ_{22} or μ_{33} obtained from VPO measurements on solutions in which either component 2 or component 3 is the only solute.

Expression for Γ_{μ_3} Based on VPO Estimation of μ_{22}. According to eq 12, knowledge of Γ_{μ_1} and Γ_{μ_2} suffices to determine Γ_{μ_3}. Over the range of solute concentrations at which Γ_{μ_1} can be quantified by VPO, corresponding values of Γ_{μ_2} cannot be determined exactly by any direct experimental method. Nevertheless, input accessible by VPO can be used to estimate the term $m_2\Gamma_{\mu_2}/m_3 = -m_2\mu_{22}/(m_3\mu_{23})$ in eq 12. For this purpose the denominator $m_3\mu_{23} = m_3\mu_{32}$ is related to the numerator $m_2\mu_{22}$ by introducing eq 10, and then μ_{22} is expressed as a sum of contributions from ideal mixing entropy and nonideality:

$$m_2\Gamma_{\mu_2}/m_3 = [m_2\mu_{22}^{\mathrm{mix}} + m_2\mu_{22}^{\mathrm{ex}}]/[m_2\mu_{22}^{\mathrm{mix}} + m_2\mu_{22}^{\mathrm{ex}} + m_1^{\bullet}\mu_{12}] \tag{14}$$

As input for eq 14, μ_{12} (or, in practice, equivalent derivatives) is determined by VPO measurements on the three-component solution and $m_2\mu_{22}^{\mathrm{mix}}$ is calculated exactly with either eq A-3a or A-3b, as appropriate for the types of solute species comprising components 2 and 3. No universally applicable expression is available for μ_{22}^{ex} as an explicit function of concentration variables and the relevant molecular parameters. Nevertheless, if the presence of solute component 3 has a negligible effect on the m_2 dependence of the macromolecular nonideality, $m_2\mu_{22}^{\mathrm{ex}}$ can be estimated as the corresponding derivative determined by using eq 8 to analyze VPO measurements on solutions in which the macromolecular component is the only solute:

$$m_2\mu_{22}^{\mathrm{ex}} \cong m_2[\mu_{22}^{\mathrm{ex}}]^{\circ(3)} = -m_1^{\bullet}[\mu_{12}]^{\circ(3)} - m_2[\mu_{22}^{\mathrm{mix}}]^{\circ(3)} \tag{15}$$

(By analogy with the symbols introduced in eqs 13a and 13b, the superscript $^{\circ(3)}$ denotes the limit $m_3 \rightarrow 0$ at fixed T, P, and $m_2 > 0$.)

Without introducing any additional approximation, $m_2\mu_{22}$, and hence $m_2\Gamma_{\mu_2}/m_3$ via eq 14, can be obtained simply by adding $m_2\mu_{22}^{\mathrm{mix}}$ to both sides of eq 15:

$$m_2\mu_{22} \cong -m_1^{\bullet}[\mu_{12}]^{\circ(3)} + m_2\delta_{22}^{\mathrm{mix}} \tag{16}$$

The term $\delta_{22}^{\mathrm{mix}} \equiv \mu_{22}^{\mathrm{mix}} - [\mu_{22}^{\mathrm{mix}}]^{\circ(3)}$ expresses the difference between ideal mixing entropy contributions to μ_{22} in the presence and absence of component 3. If components 2 and 3 dissociate with a common ion, the magnitude of $m_2\delta_{22}^{\mathrm{mix}}$ generally need not be small compared with $-m_1^{\bullet}[\mu_{12}]^{\circ(3)}$ because the ideal mixing entropy of the macromolecular component has a significant dependence on m_3 as well as m_2. For such systems, according to eq A-3b:

$$m_2\delta_{22}^{\mathrm{mix}}/(RT) \cong -\nu_+|Z_{\mathrm{M}}|m_3/(|Z_{\mathrm{M}}|m_2 + \nu_+m_3) \tag{17}$$

If components 2 and 3 do not have a common ion, $m_2\delta_{22}^{\mathrm{mix}}$, as evaluated using eq A-3a, typically is negligible compared with $-m_1^{\bullet}[\mu_{12}]^{\circ(3)}$.

Combining eqs 12, 14, and 16 yields the first of two alternative expressions for the coefficient of primary interest:

$$\Gamma_{\mu_3} \cong \frac{\Gamma_{\mu_1} + (m_1^{\bullet}\mu_{12}^{\circ(3)} - m_2\delta_{22}^{\mathrm{mix}})/(m_1^{\bullet}\mu_{13})}{1 + (m_2/m_3)(\Gamma_{\mu_1} + (m_1^{\bullet}\mu_{12}^{\circ(3)} - m_2\delta_{22}^{\mathrm{mix}})/(m_1^{\bullet}\mu_{13}))} \tag{18}$$

This equation requires as input three derivatives that can be evaluated by VPO (Γ_{μ_1}, μ_{13}, and $\mu_{12}^{\circ(3)}$) and the term $m_2\,\delta_{22}^{\mathrm{mix}}$, which can be evaluated with eq 17 if components 2 and 3 have a common ion and can be neglected (in comparison with $-m_1^{\bullet}[\mu_{12}]^{\circ(3)}$) for all other classes of three-component solutions. For practical reasons dictated by sample preparation techniques, experimental determinations[2,3,8] of the dependence of Osm on m_3 (or C_3) have been conducted at fixed molarity, rather than molality, of the macromolecular component. Results of such VPO measurements can be analyzed to quantify $m_1^{\bullet}\mu_{13}$ with expressions given by Zhang et al.[2] In eq 18, μ_{12} has been eliminated (using eq 1b) because direct evaluations of this derivative by VPO generally are subject to greater uncertainty than evaluation of the equivalent (by eq 1b) product $-(\Gamma_{\mu_1}\mu_{13})$. Although the magnitude of μ_{12} significantly exceeds that of μ_{13} under most conditions, the uncertainty in the latter derivative is lower because the range of m_3 over which Osm can be measured typically is as much as one hundred times wider than the range of m_2 (restricted by solubility).

Expression for Γ_{μ_3} Based on VPO Estimation of μ_{33}. Instead of using eqs 12 and 14 with values of μ_{22}^{ex} approximated by osmometric titrations on solutions in which component 2 is the only solute, an alternative way of calculating Γ_{μ_3} is based on the application of eqs 3b and 11 to analyze values of μ_{33}^{ex} that can be approximated by osmometric titrations on solutions in which component 3 is the only solute. Specifically, the m_3 dependence of the nonideality of component 3 is approximated as follows:

$$m_3\mu_{33}^{\mathrm{ex}} \cong m_3[\mu_{33}^{\mathrm{ex}}]^{\circ(2)} = -m_1^{\bullet}\mu_{13}^{\circ(2)} - m_3[\mu_{33}^{\mathrm{mix}}]^{\circ(2)} \tag{19}$$

$$m_3\mu_{33} \cong -m_1^{\bullet}\mu_{13}^{\circ(2)} + m_3\delta_{33}^{\mathrm{mix}} \tag{20}$$

The symbols appearing in eqs 19 and 20, and their physical significances, are analogous to those in eqs 15 and 16. Evaluation of $\delta_{33}^{\mathrm{mix}}$ in eq 20 requires consideration of explicit expressions for μ_{33}^{mix} and $[\mu_{33}^{\mathrm{mix}}]^{\circ(2)}$, which follow directly from either eq A-6a or, if components 2 and 3 have a common ion, eq A-6b. Compared with $m_1^{\bullet}\mu_{13}^{\circ(2)}$, the magnitude of $m_3\delta_{33}^{\mathrm{mix}}$ always is small unless components 2 and 3 dissociate into charged species with a common ion. For such systems:

$$m_3\delta_{33}^{\mathrm{mix}}/(RT) \equiv (\mu_{33}^{\mathrm{mix}} - [\mu_{33}^{\mathrm{mix}}]^{\circ(2)})/(RT) \cong -\nu_+|Z_{\mathrm{M}}|m_2/(|Z_{\mathrm{M}}|m_2 + \nu_+m_3) \tag{21}$$

In applications of VPO to investigate preferential interactions, m_2 is in the millimolal range. Hence, over the initial (low m_3) stage of osmometric titrations using electrolyte solutes, m_2/m_3 is not small enough to warrant neglect of $m_3\delta_{33}^{\mathrm{mix}}$ in eq 20.

The approximation built into eqs 19 and 20 entails a separation of the concentration-dependent contributions to μ_3^{ex} into one having a (typically) strong m_2 dependence, due to interactions with an isolated macromolecule, and another having an m_3 dependence that can be estimated from measurements of $[\mu_3^{\mathrm{ex}}]^{\circ(2)}$ in a corresponding solution in which component 3 is the only solute. Molecular theoretical implications of this approximation can be understood by noting that the chemical potential of solute i can be expressed as a sum of terms, each an integral over a function related to the radial distribution of

some solute species around a fixed molecule of i.[35] In general, each of these additive integrals depends on the concentrations of all of the solutes. Here, the m_3 dependence of μ_3^{ex} is assumed to arise entirely from the interactions of the species comprising component 3 with each other. The same approximation for the m_3 dependence of μ_3^{ex} has been used extensively in applications of eq 7 or equivalent expressions that entail estimation of the m_3 dependence of $\ln a_3$ in solutions in which all solutes other than component 3 are highly dilute.[36] The integrated form of the same approximation also has been utilized for comparisons of experimental measurements of μ_3^{ex} with theoretical calculations of the m_2 dependent part of this term that is due to solvent-averaged Coulombic interactions of small ions with an isolated polyion.[21,37−39]

On the basis of the approximation built into eqs 19 and 20, which requires that the difference between μ_{33}^{ex} and $[\mu_{33}^{ex}]^{o(2)}$ be negligible over the range of m_2 investigated, the following independent alternative to eq 18 is derived by introducing eqs 3b and 20 into eq 11:

$$\Gamma_{\mu_3} \cong m_3/m_2 - m_3 m_1^{\bullet}\mu_{13}/m_2(m_1^{\bullet}\mu_{13}^{o(2)} - m_3\delta_{33}^{mix}) \quad (22)$$

The two derivatives, μ_{13} and $\mu_{13}^{o(2)}$ are quantified by analyzing VPO measurements on three-component and two-component solutions, respectively. Compared with $m_1^{\bullet}\mu_{13}^{o(2)}$, the magnitude of $m_3\delta_{33}^{mix}$ is negligible for all types of three-component solutions except those containing salt cations, salt anions, and a charged macromolecule at concentrations such that $|Z_M|m_2$ is not much smaller than $\nu_+ m_3$.

Comparison of the Alternative Expressions for Γ_{μ_3}. The two expressions derived above for Γ_{μ_3}, eqs 18 and 22, have distinctly different functional forms, incorporating alternative approximations (specified in eqs 16 and 20) that may be most reliable in different ranges of m_2 and m_3. A sufficient excess of m_3 over m_2 is expected to favor the accuracy of eq 22 even when the macromolecular component is not highly dilute. In contrast, eq 18 is expected to be most accurate when m_3/m_2 is relatively small, unless interactions between macromolecules in solution have thermodynamic effects that are, for example, effectively "screened" by even small amounts of component 3. As compared with eq 22, eq 18 includes more experimental information: three, rather than two, derivatives that can be determined from VPO. Uncertainties in values of Γ_{μ_3} as calculated with eq 22 depend primarily on the accuracy with which $m_1^{\bullet}\mu_{13}/(m_1^{\bullet}\mu_{13}^{o(2)} - m_3\delta_{33}^{mix})$ can be discriminated from unity, whereas uncertainties in corresponding values of Γ_{μ_3} as calculated with eq 18 depend primarily on the accuracy with which Γ_{μ_1} can be determined by the isoosmolal method. (The average experimental uncertainty in Γ_{μ_1} is ∼10%, as estimated from VPO results[3,8] for all of the solutes of which the preferential interactions with BSA have been investigated to date.)

In the absence of any independent test of the different approximations built into eqs 18 and 22, these alternative expressions for Γ_{μ_3} can be applied separately to analyze input from appropriate sets of (different) osmometric measurements over the widest ranges of solute and macromolecular concentrations accessible by VPO. When the resulting values of Γ_{μ_3} are the same (within their uncertainties), the accuracy of the two physically distinct approximations is indicated. In our VPO studies[3,8] of the preferential interactions of a wide variety of biochemical solutes with BSA, separate applications of eqs 18 and 22 were found to yield values of Γ_{μ_3} that do not differ, outside experimental uncertainties, over ranges of m_3 up to 0.4 m for the two guanidinium salts and to at least 1 m for 10 other solutes.

III-D. Physical Origins and Thermodynamic Implications of Significant Differences Between Γ_{μ_1} and Γ_{μ_3}. In section III-B, eq 12 was used to demonstrate that $\Gamma_{\mu_3} - \Gamma_{\mu_1}$ does not vanish even as $m_2 \rightarrow 0$ because μ_2^{mix} diverges logarithmically in eqs A-2a and A-2b. More generally, for solutions in which m_2 is in the millimolal range, applications of the alternative expressions (eqs 18 and 22) to analyze VPO measurements also have demonstrated significant differences between Γ_{μ_3} and Γ_{μ_1} for the preferential interactions of a wide variety of biochemical solutes with BSA.[3,8] A primary objective of this section is to identify some general characteristics of systems and conditions in which Γ_{μ_3} differs significantly from Γ_{μ_1}. For this purpose, physical factors that govern the magnitude of $(\Gamma_{\mu_1} - \Gamma_{\mu_3})/\Gamma_{\mu_1}$, as expressed by rearranging eq 12, are traced to characteristics of Γ_{μ_2} that are determined by contributions from the ideal mixing entropy and the nonideality of the macromolecular component. By this approach, we also explain why Γ_{μ_3} and Γ_{μ_1} are approximately equal in the concentrated NaCl solutions containing dilute polymeric NaDNA that have been studied by ID.[10,32]

Use of Chemical Potential Derivatives to Express $(\Gamma_{\mu_1} - \Gamma_{\mu_3})/\Gamma_{\mu_1}$. The magnitudes and solute-concentration dependences of preferential interaction coefficients such as Γ_{μ_3} (and all other thermodynamic functions related to chemical potentials) are determined at the molecular level by some combination of contributions from nonideality and ideal mixing entropy. To proceed with a general analysis of how these two physically distinct contributions affect the relationship between Γ_{μ_1} and Γ_{μ_3}, eq 12 is rearranged to express the relative difference between these coefficients as follows:

$$(\Gamma_{\mu_1} - \Gamma_{\mu_3})/\Gamma_{\mu_1} = (1 - \Gamma_{\mu_3}/\Gamma_{\mu_2})/(1 - m_3/m_2\Gamma_{\mu_2}) \quad (23a)$$

$$= \frac{(1 - (\mu_{23})^2/(\mu_{22}\mu_{33}))}{(1 + m_3\mu_{23}/(m_2\mu_{22}))} \quad (23b)$$

The second equality follows from eqs 2b and 3b in Table 1. Equations 23a and 23b are applicable to any system in which $\Gamma_{\mu_1} \neq 0$.[40]

The necessarily positive quotient $(\mu_{23})^2/(\mu_{22}\mu_{33})$ in the numerator of eq 23b is smaller than $m_3\mu_{23}/(m_2\mu_{22})$ by the factor $m_2\mu_{23}/(m_3\mu_{33}) = -m_2\Gamma_{\mu_3}/m_3$, which vanishes as $m_2 \rightarrow 0$. In fact, $m_2\Gamma_{\mu_3}/m_3$ is always less, typically much less, than unity for the various solutes of which the preferential interactions with BSA have been investigated by VPO[2,3,8] and for the solutions containing NaCl and NaDNA in which Γ_{μ_1} has been quantified[32] by analyzing ID measurements.[10] Consequently, for the purposes of the following analyses based on eq 23b, the sign and magnitude of $(\Gamma_{\mu_1} - \Gamma_{\mu_3})/\Gamma_{\mu_1}$ are considered to be controlled entirely by the corresponding characteristics of $m_3\mu_{23}/(m_2\mu_{22})$. This term is positive for an excluded solute, so that a change in any thermodynamic variable or physical factor that causes an increase in $m_3\mu_{23}/(m_2\mu_{22})$ must reduce the magnitude of $(\Gamma_{\mu_1} - \Gamma_{\mu_3})/\Gamma_{\mu_1}$. But if $m_3\mu_{23}/(m_2\mu_{22})$ is negative, as for an accumulated solute, the magnitude of this term compared with unity determines the sign of the denominator of eq 23b and hence whether the magnitude of $(\Gamma_{\mu_1} - \Gamma_{\mu_3})/\Gamma_{\mu_1}$ is larger or smaller for larger values of $|m_3\mu_{23}/(m_2\mu_{22})|$. To facilitate reference to these alternative trends, we designate solutes for which $|m_3\mu_{23}/(m_2\mu_{22})| < 1$ as "weakly" accumulated (such as urea, at least when $m_3 < 1$ m) and solutes for which $|m_3\mu_{23}/(m_2\mu_{22})| > 1$ as "strongly" accumulated (such as the two guanidinium salts in

TABLE 2: Contributions to the Normalized Difference $(\Gamma_{\mu_1} - \Gamma_{\mu_3})/\Gamma_{\mu_1}$ for Interactions of Glycerol and of Urea with BSA

		solute					
		glycerol			urea		
small solute molality, m_3 (m)	BSA molality, m_2 (m)	$\Gamma_{\mu_3}/\Gamma_{\mu_2}$	$m_3/(m_2\Gamma_{\mu_2})$	$(\Gamma_{\mu_1} - \Gamma_{\mu_3})/\Gamma_{\mu_1}$	$\Gamma_{\mu_3}/\Gamma_{\mu_2}$	$m_3/(m_2\Gamma_{\mu_2})$	$(\Gamma_{\mu_1} - \Gamma_{\mu_3})/\Gamma_{\mu_1}$
0.1	3×10^{-3}	$(2.4 \pm 0.1) \times 10^{-3}$	-0.081 ± 0.001	0.9 ± 0.1	$(8 \pm 3) \times 10^{-4}$	0.045 ± 0.001	1.05 ± 0.04
0.5	1×10^{-3}	$(3.7 \pm 0.3) \times 10^{-3}$	-0.39 ± 0.03	0.7 ± 0.2	$(1.4 \pm 0.6) \times 10^{-3}$	0.23 ± 0.04	1.3 ± 0.2
0.5	3×10^{-3}	$(1.1 \pm 0.1) \times 10^{-2}$	-0.38 ± 0.03	0.7 ± 0.1	$(4 \pm 2) \times 10^{-3}$	0.23 ± 0.04	1.3 ± 0.2
1.0	3×10^{-3}	$(2.0 \pm 0.6) \times 10^{-2}$	-0.7 ± 0.3	0.6 ± 0.2	$(8 \pm 9) \times 10^{-3}$	0.5 ± 0.2	1.9 ± 0.4

Figure 1 at sufficiently high m_3). A parallel classification is not needed for excluded solutes because when μ_{23} is positive the sign of the denominator in eq 23b cannot change.

The magnitude and solute-concentration dependence of $m_3\mu_{23}/(m_2\mu_{22})$ are determined by contributions from ideal mixing entropy and nonideality of the macromolecular component. To examine the relative importance of these physically distinct effects, each of the derivatives of μ_2 can be expressed as a sum of contributions from μ_2^{mix} and μ_2^{ex}. Equation A-3a for μ_{22}^{mix} and eq A-4a for μ_{23}^{mix} each has the same functional form in all but one of the general classes of three-component solutions specified at the beginning of the Appendix. In the exceptional, but frequently investigated, case in which components 2 and 3 have a common ion, μ_{22}^{mix} is given by eq A-3b and μ_{23}^{mix} by eq A-4b. Universally applicable exact expressions analogous to those presented in the Appendix cannot be derived to represent μ_{22}^{ex} and μ_{23}^{ex} as explicit functions of m_2, m_3, and the pertinent molecular parameters. However, inferences about characteristics of these derivatives can be drawn from analyses of the appropriate experimental information (as demonstrated in the next section) using eqs 23a, 23b, and the expressions for μ_{22}^{mix} and μ_{23}^{mix} given in the Appendix.

Effects of Solute Type and Concentration on $(\Gamma_{\mu_1} - \Gamma_{\mu_3})/\Gamma_{\mu_1}$. To illustrate the utility of eqs 23a and 23b in analyzing the origins of significant differences between Γ_{μ_1} and Γ_{μ_3}, we consider here experimental results for aqueous solutions in which component 3 is either glycerol or urea and component 2 is the sodium salt of BSA. Preferential interaction coefficients have been quantified for glycerol with BSA[3] and with various other proteins.[41] Although urea is perhaps the most widely used perturbing solute, especially as a denaturant in studies of protein unfolding, systematic quantitative information about its preferential interactions with a native protein (Γ_{μ_3} as a function of m_3) was obtained only recently.[8] At a given m_3, values of Γ_{μ_3} are similar in magnitude for glycerol and urea but negative for the excluded solute (glycerol) and positive for the weakly accumulated solute (urea). For each of these solutes at representative choices of m_2 and m_3, values of $(\Gamma_{\mu_1} - \Gamma_{\mu_3})/\Gamma_{\mu_1}$ are presented in Table 2, together with corresponding values of the terms $\Gamma_{\mu_3}/\Gamma_{\mu_2}$ and $m_3/(m_2\Gamma_{\mu_2})$ in eq 23a. These numbers were calculated by using eqs 18 and 22 to obtain consistent values of Γ_{μ_3} and eq 12 to obtain corresponding values of Γ_{μ_2}.

Because glycerol is an excluded solute, Γ_{μ_3} is (by definition) negative. Therefore, eqs 2b and 3b require that Γ_{μ_2} also is negative (because μ_{22} and μ_{33} both must be positive[13]), and hence, the denominator on the right-hand side of eq 23a must be positive. The numerator also is positive because the values of $\Gamma_{\mu_3}/\Gamma_{\mu_2}$ given in Table 2 demonstrate that the magnitude of this quotient always is small compared with unity. Consequently, eq 23a shows that the magnitude of Γ_{μ_1} (necessarily negative for any excluded solute) exceeds that of Γ_{μ_3} and the dependence of the positive quotient $(\Gamma_{\mu_1} - \Gamma_{\mu_3})/\Gamma_{\mu_1}$ on m_3 is determined entirely by $m_3/(m_2\Gamma_{\mu_2})$. The values of this term given for glycerol in Table 2 show that it (like $\Gamma_{\mu_3}/\Gamma_{\mu_2}$) increases approximately in direct proportion to the 10-fold increase in m_3, while $(\Gamma_{\mu_1} - \Gamma_{\mu_3})/\Gamma_{\mu_1}$ decreases somewhat but remains large enough so that these coefficients differ significantly at any m_3 in the range of 0.1−1.0 m. The trends in $\Gamma_{\mu_3}/\Gamma_{\mu_2}$ and $m_3/(m_2\Gamma_{\mu_2})$ exhibited by glycerol are qualitatively similar to those found for the more strongly excluded solutes of which the preferential interactions with BSA have been investigated by VPO[3] (except that for glycine betaine, when $m_3 > 0.5$, $\Gamma_{\mu_3}/\Gamma_{\mu_2}$ in eq 23a becomes large enough so that Γ_{μ_3} becomes more negative than Γ_{μ_1} and therefore the sign of $(\Gamma_{\mu_1} - \Gamma_{\mu_3})/\Gamma_{\mu_1}$ changes from positive to negative).

For the accumulated solute urea, Γ_{μ_2}, like Γ_{μ_3}, is positive. As in the case of glycerol, the results in Table 2 show that a 10-fold increase in m_3 produces an approximately proportional change in both $\Gamma_{\mu_3}/\Gamma_{\mu_2}$ and $m_3/(m_2\Gamma_{\mu_2})$. Again, because $\Gamma_{\mu_3}/\Gamma_{\mu_2}$ remains much less than one, $m_3/(m_2\Gamma_{\mu_2})$ in effect controls the sign, magnitude, and m_3 dependence of $(\Gamma_{\mu_1} - \Gamma_{\mu_3})/\Gamma_{\mu_1}$. Over the entire range of urea concentrations investigated, the increasingly positive values of $m_3/(m_2\Gamma_{\mu_2})$ do not exceed unity. Consequently, $(\Gamma_{\mu_1} - \Gamma_{\mu_3})/\Gamma_{\mu_1}$, again positive, increases with increasing m_3, in contrast to the trend exhibited by glycerol. As m_3 increases 10-fold, $(\Gamma_{\mu_1} - \Gamma_{\mu_3})/\Gamma_{\mu_1}$ for urea increases by approximately a factor of 2; therefore, at least over this range of m_3, Γ_{μ_3} cannot be approximated by Γ_{μ_1}. The absolute magnitudes of these coefficients are similar to those for glycerol at the same concentrations.[1,8] Although Γ_{μ_1} remains negative for urea over the range of m_3 investigated, in accordance with eqs 10 and 11, Γ_{μ_1} can be positive for an accumulated solute. (For example, the strongly favorable preferential interactions with BSA of the chloride and thiocyanate salts of guanidinium cause the sign of Γ_{μ_1} to change from negative to positive with increasing m_3. Our VPO results for the preferential interactions of these strongly accumulated solutes with BSA are analyzed and interpreted elsewhere.[8])

According to eq 23b, the experimental results presented in Table 2 have the following implications concerning the relative magnitudes of the derivatives μ_{23}, μ_{22}, and μ_{33} that determine $(\Gamma_{\mu_1} - \Gamma_{\mu_3})/\Gamma_{\mu_1}$. For both urea and glycerol over the entire range of concentrations examined, the numerical insignificance of $(\mu_{23})^2/(\mu_{22}\mu_{33})$ compared with unity is consistent with the inference that μ_{22} is substantially larger than μ_{23} and consistent with the expectation that the interactions of nonelectrolyte solutes such as these with a macromolecular protein like BSA are relatively weak (as compared, for example, with the interactions of the zwitterion glycine betaine with BSA[3]). For both glycerol and urea, the insensitivity of $m_3\mu_{23}/(m_2\mu_{22})$ to a 3-fold change in m_2 at $m_3 = 0.5$ indicates that neither μ_{23} nor $m_2\mu_{22}$ depends significantly on m_2. Hence, either μ_{22} is completely dominated by μ_{22}^{mix}, or μ_{22}^{ex} is proportional to $1/m_2$. For both urea and glycerol, values of μ_{23} can be estimated[42] on the basis of the experimental information in Table 2 and Figure 1 and the approximation for μ_{33} specified in eq 20. Compared with these estimated values of μ_{23}, the corresponding values of μ_{23}^{mix}, calculated exactly by eq A-4a, are minuscule over the concentration ranges represented in Table 2. Therefore, for both urea and glycerol, the magnitude of $(\Gamma_{\mu_1} - \Gamma_{\mu_3})/\Gamma_{\mu_1}$ is determined primarily by $m_3\mu_{23}^{ex}/(m_2\mu_{22})$. The sign of this quo-

tient (negative for glycerol, positive for urea) is determined by μ_{23}^{ex} because (for the reason given in section II-A) μ_{22} must be positive.

As indicated by the results presented in Table 2 for both glycerol and urea, the approximately 10-fold change in $m_3\mu_{23}^{ex}/(m_2\mu_{22})$ over a 10-fold change in m_3 can be accounted for entirely by the explicit factor m_3, which originates in the Gibbs−Duhem linkage expressed by eq 10. The absence of any significant dependence of μ_{23}^{ex} on m_3 for either glycerol or urea implies that in each case μ_2^{ex} must be approximately proportional to m_3. These constants of proportionality are of similar magnitude. The foregoing conclusions concerning effects on $(\Gamma_{\mu_1} - \Gamma_{\mu_3})/\Gamma_{\mu_1}$ due to characteristics of $m_3\mu_{23}^{ex}/(m_2\mu_{22})$ are also completely consistent with quantitative analyses of the solute-concentration dependence of Γ_{μ_3} based on the local-bulk domain model for glycerol[3] and for urea.[8]

Effects of the Net Macromolecular Charge on the Magnitude of $(\Gamma_{\mu_1} - \Gamma_{\mu_3})/\Gamma_{\mu_1}$ for Various General Classes of Small Solute Components. The results presented in Figure 1 for high macromolecular dilutions and in Table 2 for m_2 in the millimolal range illustrate how the difference between Γ_{μ_3} and Γ_{μ_1} for a given macromolecule is affected by individual characteristics of various kinds of solutes. For any particular small solute in a solution with composition specified by m_3 and m_2, $|(\Gamma_{\mu_1} - \Gamma_{\mu_3})/\Gamma_{\mu_1}|$ also depends on the macromolecular charge and on other general physical characteristics of the macromolecule, in particular the amount of its surface area that is accessible for interactions with solvent and the small solute. In this section, we examine how a variation in the net macromolecular charge, $|Z_M|$, affects $|(\Gamma_{\mu_1} - \Gamma_{\mu_3})/\Gamma_{\mu_1}|$ in otherwise equivalent three-component solutions. For this purpose, we utilize the explicit expressions for μ_{22}^{mix} and μ_{23}^{mix} given in the Appendix (eqs A-3a, A-3b, A-4a, and A-4b) and consider systems, such as polymeric DNA in solutions containing a sufficient excess of NaCl, in which μ_{23}^{ex} is a monotonic function of the (net) number of charges on the macromolecule and the $|Z_M|$ dependence of μ_{22}^{ex} is negligible.

In general, the $|Z_M|$ dependence of $|(\Gamma_{\mu_1} - \Gamma_{\mu_3})/\Gamma_{\mu_1}|$ is determined by contributions from ideal mixing entropy and nonideality to the three derivatives of chemical potentials specified in eq 23b. If the distribution of structural charges on the macromolecule is sufficiently regular so that virtually all of them have thermodynamically equivalent interactions with the small solute ions, then μ_2^{ex} is expected to increase monotonically in direct proportion to $|Z_M|$, at least when the macromolecule is dilute enough so that its nonideality is determined by Coulombic interactions with the ions that comprise component 3, rather than with other macromolecules (and provided that any differences in macromolecular size implied by differences in $|Z_M|$ make no significant contribution to the macromolecular nonideality due to excluded volume). Thus, the magnitude and $|Z_M|$ dependence of the necessarily positive derivative μ_{22} are determined, under the conditions considered here, by μ_{22}^{mix}, while the magnitude and sign of μ_{23} are determined by μ_{23}^{ex} and, if components 2 and 3 have an ion in common, by μ_{23}^{mix}.

To draw general inferences about the physical origins of the $|Z_M|$ dependence of $(\Gamma_{\mu_1} - \Gamma_{\mu_3})/\Gamma_{\mu_1}$, we consider solutions that are in the same thermodynamic state (specified by T, P, m_2, and m_3), in which the small solute species are chemically identical and the macromolecular species differ only in $|Z_M|$, with regard to characteristics that can affect preferential interactions. As indicated by the following summary statements, the macromolecular charge affects μ_{22}^{mix}, μ_{23}^{mix}, and μ_{23}^{ex} in markedly different ways, depending on whether the solute species comprising component 3 are charged and, if so, whether one of the dissociated solute ions is the same as the macromolecular counterion.

(1) *If component 3 consists of uncharged molecules*, like urea or glycerol, μ_{23}^{ex} has no $|Z_M|$ dependence due to Coulombic interactions. If the solute−macromolecule preferential interactions are strong enough (as they are for glycerol and urea) so that the magnitude of μ_{23}^{ex} predominates over that of μ_{23}^{mix}, then the $|Z_M|$ dependence of μ_{23}^{mix}, as given by eq A-4a, is insignificant compared with the $|Z_M|$ dependence of μ_{22}^{mix}, as given by eq A-3a. **In such systems the magnitude of $(\Gamma_{\mu_1} - \Gamma_{\mu_3})/\Gamma_{\mu_1}$ increases with increasing $|Z_M|$ for an excluded or strongly accumulated small solute; this trend is reversed for a weakly accumulated small solute**.

(2) *If components 2 and 3 dissociate but have no ion in common*, the $|Z_M|$ dependence of μ_{23}^{ex} that arises from Coulombic interactions of salt ions with the macromolecular structural charges (together with the $|Z_M|$ dependence of μ_{23}^{mix} given by eq A-4a, if this term is not entirely negligible) is opposed by the $|Z_M|$ dependence of μ_{22}^{mix} given by eq A-3a. **In such systems, the magnitude of $(\Gamma_{\mu_1} - \Gamma_{\mu_3})/\Gamma_{\mu_1}$ is relatively insensitive to changes in $|Z_M|$, regardless of whether the small solute component is excluded or accumulated**.

(3) *If components 2 and 3 dissociate with a common ion*, comparison of eqs A-3a and A-3b shows that the contribution to μ_{22}^{mix} from the ideal mixing entropy of the macromolecular counterions is damped by the factor

$[|Z_M|m_2/(|Z_M|m_2 + \nu_+m_3)]$. When this quotient is sufficiently small compared with unity, the $|Z_M|$ dependence of $m_3\mu_{23}/(m_2\mu_{22})$ in eq 23 is determined predominantly by $\mu_{23}^{ex} + \mu_{23}^{mix}$. **In such systems, the magnitude of $(\Gamma_{\mu_1} - \Gamma_{\mu_3})/\Gamma_{\mu_1}$ decreases with increasing $|Z_M|$ for an excluded or strongly accumulated small solute component; this trend is reversed for a weakly accumulated small solute component.**

Exceptional Case of a System in Which $(\Gamma_{\mu_1} - \Gamma_{\mu_3})/\Gamma_{\mu_1}$ is Negligible. According to the third alternative situation specified above, if the solution contains a highly charged macromolecule and a sufficient excess of some salt with an ion of the same type as the macromolecular counterion, μ_{22}^{mix} in the denominator of $m_3\mu_{23}/(m_2\mu_{22})$ has no dependence on $|Z_M|$ to counteract the $|Z_M|$ dependences of μ_{23}^{ex} and of μ_{23}^{mix} in the numerator. As a consequence of this common ion effect, especially when m_2 is dilute and m_3 concentrated, eq 23b indicates that the numerical difference between Γ_{μ_1} and Γ_{μ_3} could be negligible. Approximate equality of these coefficients is implied by an analysis[7,32] of experimental information[9,10] for the system described below. Because citations of this important example are widespread in the literature, they may promote the erroneous impression that Γ_{μ_1} and Γ_{μ_3} generally are numerically indistinguishable. However, the following analysis indicates that significant differences between Γ_{μ_1} and Γ_{μ_3} generally are to be expected for the preferential interactions of a small solute with a macromolecule.

Prior to the VPO studies of solute−protein preferential interactions reported from this laboratory,[2,3,8] an experimentally based thermodynamic analysis of relationships among preferential interaction coefficients in a three-component system was available (to the best of our knowledge) only for aqueous solutions containing the sodium salt of polymeric native DNA and high (>1 M) concentrations of NaCl. For this system, ID

measurements[10] were reanalyzed[32] to obtain values of Γ_{μ_1} that were compared with corresponding values of Γ_{μ_1,μ_3} by utilizing a thermodynamic relationship that incorporates van't Hoff's Law (to approximate the "osmotic pressure" difference between salt−DNA and salt solutions equilibrated by dialysis) and an approximation for μ_{33} equivalent to our eq 20. This analysis (summarized on pp 45−46 of ref 7) indicates negligible numerical differences between Γ_{μ_1,μ_3} and Γ_{μ_1}. Application of a similar approach to the same system and conditions (p 42 of ref 7) shows that $\Gamma_{\mu_1,\mu_3} \cong \Gamma_{\mu_3}$. These separate comparisons of Γ_{μ_1,μ_3} with Γ_{μ_1} and with Γ_{μ_3} imply that $\Gamma_{\mu_1} \cong \Gamma_{\mu_3}$.

The discussion in the preceding section of effects of $|Z_M|$ on the magnitude of $(\Gamma_{\mu_1} - \Gamma_{\mu_3})/\Gamma_{\mu_1}$ for different classes of three-component solutions, in conjunction with the trends exhibited in Table 2, provides a qualitative basis for understanding why, in solutions containing NaCl and the sodium salt of polymeric DNA (at least under the conditions in which the ID measurements were made[10]), relationships linking different preferential interaction coefficients have characteristics that differ substantially from those generally expected for other three-component solutions containing small solutes and a biopolymer. For solutions such as the DNA−salt systems studied by isopiestic distillation,[10] eq 23b shows that $|(\Gamma_{\mu_1} - \Gamma_{\mu_3})/\Gamma_{\mu_1}|$ is smaller when the number of equivalent structural charges on the macromolecule is larger because both μ_{23}^{ex} and μ_{23}^{mix} (as given by eq A-4b) scale with $|Z_M|$ whereas μ_{22}^{mix} has no significant $|Z_M|$ dependence when components 2 and 3 have an ion in common. Furthermore, when the concentration of an excluded solute component (like NaCl) is higher, the magnitudes of both $-(\mu_{23})^2/(\mu_{22}\mu_{33})$ and $m_3\mu_{23}/(m_2\mu_{22})$ (intrinsically positive for an excluded solute) are larger and hence, because of the placement of these terms in eq 23b, the magnitude of $(\Gamma_{\mu_1} - \Gamma_{\mu_3})/\Gamma_{\mu_1}$ is smaller.

In the BSA solutions investigated by VPO,[3,8] none of the dissociated salts has an ion in common with the macromolecular component and none of the small solute concentrations is as high as the lowest salt concentration in the NaCl−NaDNA solutions for which ID measurements were reported.[10] Furthermore, for the preferential interactions of BSA with KCl (or any of the other solutes investigated[3,8] by VPO that exhibit significant differences between Γ_{μ_1} and Γ_{μ_3}), the magnitudes of Γ_{μ_3}/m_3 are substantially smaller than are those for the preferential interactions of NaCl with polymeric DNA, primarily because the net charge on BSA is smaller by at least 2 orders of magnitude than the total number of phosphate charges on the native DNA investigated by ID.[10]

The foregoing considerations indicate why the term $m_3\mu_{23}/(m_2\mu_{22})$ in the denominator of eq 23b is smaller, and hence $|(\Gamma_{\mu_1} - \Gamma_{\mu_3})/\Gamma_{\mu_1}|$ larger, in BSA solutions as compared with those containing polymeric DNA. Nevertheless, the significant numerical differences between Γ_{μ_3} and Γ_{μ_1} found for solute−BSA preferential interactions do not conflict with the well-known analysis[7] of salt−DNA preferential interactions under the conditions investigated by ID.[10] For that system, the approximate equality of $\Gamma_{\mu_1} \cong \Gamma_{\mu_3}$ can be understood, on the basis of eq 23b, to result in part from the high salt concentration (>1.5 M) but primarily from the effect of the large number of phosphate charges ($|Z_M| \simeq 2000$) on μ_{23}, which is not counteracted by μ_{22} because the $|Z_M|$ dependence of this derivative is eliminated by the common ion effect. Consequently, the magnitude of $m_3\mu_{23}/(m_2\mu_{22})$ in eq 23b is large enough to reduce the relative difference between Γ_{μ_1} and Γ_{μ_3} below the threshold of experimental detectability. More generally, however, for solutions containing less highly charged biopolymers, such as proteins, and small solutes having no ion in common with the macromolecular component at concentrations in the typical physiological range, eq 23b indicates why significant differences between Γ_{μ_1} and Γ_{μ_3} are to be expected. The existence of these differences motivates the development in this paper of a rigorous thermodynamic basis for analyzing the VPO measurements with which preferential interaction coefficients can be quantified.

Acknowledgment. We thank Dan Felitsky and Jiang Hong of this laboratory for their comments on the manuscript and Sheila Aiello for assistance in preparing it for publication. The remarks of Dr. Henryk Eisenberg and anonymous referees provided helpful guidance in revision. This research was supported by NIH Grant GM 47022 (MTR).

Appendix: Contributions of Ideal Mixing Entropy to Solute Component Chemical Potentials and their Derivatives in Various General Classes of Three-Component Solutions

Three-Component Solutions Classified by Characteristics of the Constituent Species. The coefficients defined in Table 1 characterize thermodynamic effects of preferential interactions in any solution that contains two independently variable solute components. Each of these can consist either of molecules with no net charge or of an electroneutral combination of dissociated ions. In this paper, we consider only systems in which neither of the solute components is comprised of more than two dissociated species and in which no concentration-dependent processes (such as aggregation or complexation) produce solute species at concentrations that cannot be varied independently. With these provisos, a three-component solution can contain either two solute species—each with no net charge, three solute species—one uncharged and the other two comprising a fully dissociated electroneutral "binary" component, three charged solute species—if components 2 and 3 both are binary and have a common ion, or four charged solute species, which are pairwise electroneutral in the sense that the total charge due to each type of cationic species is exactly compensated by the total charge due to one of the anionic species, as in any solution prepared by dissolving arbitrary amounts of two binary components such as NaDNA and KCl. (If the four charged solute species are not pairwise electroneutral, the solution contains three independently variable binary solute components. Preferential interactions in such systems are characterized by coefficients that differ from any of those defined in Table 1.)

The classification of three-component solutions specified above is required to analyze how the coefficients defined in Table 1, and relationships among them, are affected by contributions from the ideal mixing entropy of the various kinds of species, charged or uncharged, that can comprise components 2 and 3. Specifically, in this Appendix, we show that differences in the contributions from ideal mixing entropy cause μ_{22}, μ_{23}, and μ_{33} to have significantly different dependences on m_2 and m_3 in solutions in which components 2 and 3 have a common ion, as contrasted with any of the other classes of three-component solutions. These expressions are used both in section III-C, in our derivations of alternative expressions for Γ_{μ_3}, and in section III-D to examine how the presence of a common ion affects the relative difference between Γ_{μ_3} and Γ_{μ_1} given by eq 23b.

Physical Origins of the Concentration Dependences of μ_2 and μ_3. For each of the solute species *i* comprising components

2 and 3, the concentration-dependent part of μ_i can be represented as $\mu_i^{mix} + \mu_i^{ex}$. The contribution from ideal mixing entropy is $\mu_i^{mix} \equiv RT \ln x_i$, where x_i denotes the mole fraction of species i. The "excess" chemical potential, μ_i^{ex}, represents the nonideality that results because the interactions of i with all of the solute species (including itself) differ from its interactions with solvent water. In an ideal dilute solution (as conventionally defined), $\mu_i^{ex} = 0$ regardless of the concentrations of the constituent species. In a corresponding real solution at typical experimental concentrations, the thermodynamic consequences of intersolute interactions generally are so complex that none of the μ_i^{ex} can be expressed explicitly as a function of concentration variables and the pertinent molecular parameters. For typical systems and conditions in which preferential interactions have been investigated, μ_2^{ex} has a substantial m_3 dependence due to preferential interactions of the smaller solute component with the macromolecule. As explained in comments following eq 13a, the m_2 dependence of μ_2^{ex} is negligible at sufficiently high macromolecular dilutions; however, if nonideality due to macromolecular "crowding" is significant, μ_{22}^{ex} may not be small compared with μ_{22}^{mix}.

For each uncharged species i and each electroneutral component comprised of two species, the chemical potential is determined (except for an arbitrary additive constant) by the interactions and ideal mixing entropy of all of the chemically distinct species in the system. However, detectable changes in the chemical potentials of species or components at constant T and P can be caused only by changing the concentration of one (or more) of the independently variable **components**. Therefore, the preferential interaction coefficients in eqs 1b, 2b, and 3b (Table 1) are represented as quotients of derivatives of component chemical potentials with respect to component molalities (as defined in the footnote to Table 1). A change in the molality of a binary component produces changes in the molalities of both constituent species. Accordingly, to express contributions from ideal mixing entropy to μ_2, μ_3, and their derivatives with respect to m_2 and m_3, the mole fraction of each of the solute species comprising components 2 and 3 must be expressed in terms of the component molalities $m_1^{\bullet}$, m_2, and m_3.

The ideal mixing entropy of each chemically distinct charged species in a solution can be changed only by changing the molality of one (or more) of the independently variable components. Therefore, $\ln x_i$ is represented as $\ln(m_i/\sigma_m)$, where the sum of molalities of all chemically distinct species is as follows:

$$\sigma_m \equiv m_1^{\bullet} + (1 + |Z_M|)m_2 + \nu m_3 \qquad \text{(A-1)}$$

Here $|Z_M|$ is the net charge, if any, on the macromolecule. To simplify notation in the expressions presented below Z_M is taken to be negative, as is the case for most proteins and all nucleic acids. In systems in which the perturbing solute is a molecule with no net charge, $\nu = 1$. If component 3 dissociates completely into cations with charge z_+ and anions with charge z_-, then $\nu = \nu_+ + \nu_-$, where $\nu_+ \equiv |z_-|$ and $\nu_- \equiv z_+$. Although eq A-1 pertains to any of the general classes of three-component solutions specified above, its form has been simplified by taking the macromolecular counterions to be univalent cations. (Otherwise, the factor $|Z_M|$ must be divided by the counterionic charge. Regardless of the charge on the macromolecular counterions, the general symbol ν_- is retained in the following expressions for the number of anions dissociated from component 3 in solutions in which components 2 and 3 have a common ion.)

Numerical differences between σ_m and $m_1^{\bullet}$, the constant water "molality", are small under typical experimental conditions. On this basis, the contributions to σ_m from m_2 and m_3 commonly are neglected, for example, when the activity coefficient of a solute species i is defined with respect to its molality, rather than to x_i, as an approximate separation of the contribution of nonideality to μ_i from the contribution due to ideal mixing entropy.[11] However, this approximation does not necessarily imply that derivatives of σ_m with respect to m_2 or m_3 always are small, either absolutely or in comparison to other terms that must be taken into account for the purpose of examining how contributions from ideal mixing entropy affect the magnitudes and concentration dependences of preferential interaction coefficients. Consequently, in the following expressions for contributions from ideal mixing entropy, terms arising from $\ln(\sigma_m/m_1^{\bullet})$ are not neglected a priori, and the (generally valid) justifications for neglecting such terms are shown not always to follow simply from the smallness of m_2 and m_3 compared with $m_1^{\bullet}$.

Common Ion Effects on Contributions of Ideal Mixing Entropy to μ_2, μ_3 and Their Derivatives. This section presents expressions for μ_2^{mix}, μ_3^{mix}, and their three independent derivatives, μ_{22}^{mix}, μ_{23}^{mix}, and μ_{33}^{mix}, nondimensionalized with respect to RT, as explicit functions of m_2, m_3, and $|Z_M|$. Each of these five expressions has two forms that, for most systems and conditions, differ significantly. The first (denoted "a") pertains to all the general classes of three-component solutions specified above **except** those in which components 2 and 3 have a common ion, to which the second form (denoted "b") pertains. In commenting on the expressions given for μ_{22}^{mix}, μ_{23}^{mix}, and μ_{33}^{mix}, we consider the justifiability of approximating mole fractions by the corresponding molalities (thus neglecting contributions from $\ln(\sigma_m/m_1^{\bullet})$) and summarize the physical significance of each of these derivatives as they appear in various expressions derived in this paper. In particular, we note the substantial entropic effects that arise when components 2 and 3 have a common ion.

The contribution of ideal mixing entropy to the chemical potential of the macromolecular component has the following alternative forms:

$$\mu_2^{mix}/(RT) = \ln[m_2/\sigma_m] + |Z_M| \ln[|Z_M|m_2/\sigma_m] \quad \text{(A-2a)}$$

$$\mu_2^{mix}/(RT) = \ln[m_2/\sigma_m] + |Z_M| \ln[(|Z_M|m_2 + \nu_+ m_3)/\sigma_m] \quad \text{(A-2b)}$$

The common ion effect on $\mu_2^{mix}/(RT)$, and hence $\mu_2/(RT)$, can be expressed explicitly by comparing eq A-2a for a three-component solution consisting of four pairwise electroneutral charged species (a macroion, small anion and two types of univalent cations) with eq A-2b for an equivalent solution in which only one type of univalent cation is present. In both solutions, m_2, m_3, ν, and $|Z_M|$ all are the same. Subtraction of eq A-2a from eq A-2b shows that the commingling of indistinguishable cations from components 2 and 3 raises (makes less negative) μ_2^{mix} by $|Z_M| \ln[1 + \nu_+ m_3/(|Z_M|m_2)]$. This substantial thermodynamically unfavorable common ion effect becomes more pronounced with a larger net macromolecular charge and a larger excess of m_3 over m_2.

For each distinguishable class of three-component solution, the m_2 dependence of μ_2^{mix} is expressed by one of the following derivatives:

$$\mu_{22}^{mix}/(RT) = (1 + |Z_M|)/m_2 - (1 + |Z_M|)^2/\sigma_m \cong (1 + |Z_M|)/m_2 \quad \text{(A-3a)}$$

$$\mu_{22}^{mix}/(RT) = 1/m_2 + |Z_M|^2/(|Z_M|m_2 + \nu_+ m_3) - (1 + |Z_M|)^2/\sigma_m \cong 1/m_2 + |Z_M|^2/(|Z_M|m_2 + \nu_+ m_3) \quad \text{(A-3b)}$$

For highly charged macromolecules, $(1 + |Z_M|)^2/\sigma_m$ clearly is not an intrinsically small number. Neglect of this term is justified in eq A-3a when $(1 + |Z_M|)m_2 \ll m_1^\bullet$, as in most studies of solute−macromolecule preferential interactions or their thermodynamic effects on processes. Neglect of $(1 + |Z_M|)^2/\sigma_m$ in eq A-3b entails the more stringent requirement that $(1 + |Z_M|)^2 m_2 \ll m_1^\bullet$, which may not be fulfilled for highly charged macromolecules at concentrations accessible to experimental study.

Equations A-3a and A-3b both show that an increase in m_2 raises $\mu_2^{mix}/(RT)$, but this thermodynamically unfavorable entropic effect is dramatically diminished by the presence of a common ion. Specifically, comparison of the approximate forms of eqs A-3a and A-3b for two solutions that are equivalent (as specified above) shows that in the solution containing only one type of cation its contribution to $\mu_{22}^{mix}/(RT)$ is reduced by the factor of $m_2|Z_M|/[(|Z_M|m_2 + \nu_+ m_3)]$. This quotient is smaller when the excess of m_3 over m_2 is larger and vanishes as $m_2 \rightarrow 0$. Hence, in this limit, the presence of a common ion has the maximum effect on the relationship between Γ_{μ_3} and Γ_{μ_1}. Comparison of eqs 13a and 13b shows that $\Gamma_{\mu_3}^{\circ(2)} - \Gamma_{\mu_1}^{\circ(2)}$ is reduced by a factor of $(1 + |Z_M|)^{-1}$. This substantial common ion effect is illustrated in Figure 1 by the comparison of the m_3 dependences of $\Gamma_{\mu_3}^{\circ(2)} - \Gamma_{\mu_1}^{\circ(2)}$ for NaCl and KCl.

Whenever the magnitudes of $|Z_M|$ and $|Z_M|m_2/m_3$ are such that $m_2|Z_M|/[(|Z_M|m_2 + \nu_+ m_3)] \ll 1$, then $m_2\mu_{22}^{mix}/(RT) \cong 1$, as in solutions in which the macromolecule is uncharged. This entropic damping effect was discussed in section III-D, in which contributions from ideal mixing entropy to terms in eq 23b were shown to account for the substantial reduction in $(\Gamma_{\mu_1} - \Gamma_{\mu_3})/\Gamma_{\mu_1}$ produced by the presence of a common ion. As an alternative way of expressing the common ion effect on $\mu_{22}^{mix}/(RT)$, subtraction of eq A-3a from A-3b gives $-|Z_M|\nu_+ m_3/[m_2(|Z_M|m_2 + \nu_+ m_3)]$. The same term ($m_2\delta_{22}^{mix}$ in eq 17) appears in eq 18 for Γ_{μ_3}, in which it makes a nonnegligible contribution that increases steadily as m_3 increases over the range typically investigated in VPO experiments.

The derivative expressing the m_3 dependence of μ_2^{mix}, which by Euler reciprocity is identical to the derivative expressing the m_2 dependence of μ_3^{mix}, has the following alternative forms, corresponding to eqs A-2a and A-2b:

$$\mu_{23}^{mix}/(RT) = -(1 + |Z_M|)\nu/\sigma_m \quad \text{(A-4a)}$$

$$\mu_{23}^{mix}/(RT) = |Z_M|\nu_+/(|Z_M|m_2 + \nu_+ m_3) - (1 + |Z_M|)\nu/\sigma_m \cong |Z_M|\nu_+/(|Z_M|m_2 + \nu_+ m_3) \quad \text{(A-4b)}$$

The negative sign in eq A-4a implies that an increase in m_3 has a thermodynamically favorable effect on μ_2^{mix}. If x_2 were approximated by m_2, the resulting estimate of μ_{23}^{mix} would be zero. In fact, this derivative is intrinsically small only when $|Z_M|$ is minimal. Especially for highly charged polymeric nucleic acids, μ_{23}^{mix} need not be negligible compared to μ_{23}^{ex} when the small solute is uncharged or when it is an electrolyte at such high concentrations that the Coulombic contribution to μ_{23}^{ex} is small (because of screening).

In striking contrast to the strong diminution in μ_{22}^{mix} produced by the presence of a common ion, the common ion effect on μ_{23}^{mix}, as exhibited by comparison of eqs A-4a and A-4b, considerably enhances the m_3 dependence of μ_2^{mix} and causes it to change sign under typical experimental conditions, in which $(|Z_M|m_2 + \nu_+ m_3) \ll m_1^\bullet$. Thus, as an entropic consequence of the presence of a common ion, the magnitude of μ_{23}^{mix} generally cannot be neglected in comparison to μ_{23}^{ex}, even for systems and conditions in which the m_3 dependence of macromolecular nonideality is strong, as in solutions in which salt ions interact with the high density of phosphate charges on a polymeric nucleic acid.

The contribution of ideal mixing entropy to the chemical potential of the small solute component has the following alternative forms:

$$\mu_3^{mix}/(RT) = \nu_+ \ln[\nu_+ m_3/\sigma_m] + \nu_- \ln[\nu_- m_3/\sigma_m] \quad \text{(A-5a)}$$

$$\mu_3^{mix}/(RT) = \nu_+ \ln[(|Z_M|m_2 + \nu_+ m_3)/\sigma_m] + \nu_- \ln[\nu_- m_3/\sigma_m] \quad \text{(A-5b)}$$

Under typical experimental conditions (when $|Z_M|m_2 \ll \nu_+ m_3$), the thermodynamically unfavorable common ion effect on $\mu_3^{mix}/(RT)$, obtained by subtracting eq A-5b from eq A-5a, is much smaller (approximately $|Z_M|m_2/m_3$) than the difference between eqs A-2a and A-2b for $\mu_2^{mix}/(RT)$. Differentiation of eqs A-5a and A-5b with respect to m_3 gives the following:

$$\mu_{33}^{mix}/(RT) = \nu/m_3 - \nu^2/\sigma_m \cong \nu/m_3 \quad \text{(A-6a)}$$

$$\mu_{33}^{mix}/(RT) = \nu_+^2/(|Z_M|m_2 + \nu_+ m_3) + \nu_-/m_3 - \nu^2/\sigma_m \cong \nu_+^2/(|Z_M|m_2 + \nu_+ m_3) + \nu_-/m_3 \quad \text{(A-6b)}$$

The term neglected in the approximate forms of these equations is not inherently close to zero ($\nu^2/\sigma_m \cong 0.07$ for a 1:1 electrolyte at $m_3 \simeq 1m$). However, the approximations $\nu m_3 \ll m_1^\bullet$ and $\nu^2 m_3 \ll \nu_+ m_1^\bullet$ in eqs A-6a and A-6b, respectively, are numerically inconsequential over the range of m_3 in which VPO can be used to quantify preferential interaction coefficients.

According to either eq A-6a or eq A-6b, an increase in m_3 raises $\mu_3^{mix}/(RT)$. Comparison of these equations shows that this thermodynamically unfavorable entropic contribution to the m_3 dependence of μ_3 is reduced by the presence of a common ion. This common ion effect can be quantified by subtracting eq A-6a from eq A-6b to obtain $-\nu_+|Z_M|m_2/[m_3(|Z_M|m_2 + \nu_+ m_3)]$, which becomes negligible when $m_3/(|Z_M|m_2)$ is sufficiently large and vanishes completely in the limit $m_2 \rightarrow 0$. Thus, in contrast to the substantial common ion effect on $m_2\mu_{22}^{mix}$ exhibited by comparing eqs 13a and 13b for $\Gamma_{\mu_3}^{\circ(2)} - \Gamma_{\mu_1}^{\circ(2)}$, the factor $m_3(\mu_{33}^{mix})^{\circ(2)}$ in these equations is completely unaffected by the presence of a common ion. However, in the initial stage of an osmometric titration, the magnitude of the correction term $m_3\delta_{33}^{mix}$, defined in eq 21, is not intrinsically small in solutions in which components 2 and 3 have a common ion and therefore may not be negligible when eq 22 is used to evaluate Γ_{μ_3} for such systems.

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