# Uncovering the Basis for Nonideal Behavior of Biological Molecules†

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*Received June 24, 2004; Revised Manuscript Received September 4, 2004*

ABSTRACT: The molecular origin of the nonideal behavior for concentrated binary solutions of biochemical compounds is examined. The difference between activities expressed in the molar and molal conventions can be large. Considering the range from dilute to concentrated, we show that molar activity coefficients can be represented by simple but rigorous equations involving between one and three parameters only. We derive a universal relationship interconverting the scales of molarity and molality without requiring the density of the solution. The equations are developed from first principles using a statistical thermodynamic theory of molar activity coefficients. It is shown how to express activity coefficients in different concentration scales, and the advantages and disadvantages of using certain scales are discussed and compared with the experimental data. Several classes of biochemically relevant compounds, many of which are naturally occurring osmolytes, are discussed: six saccharides (glucose, xylose, maltose, mannose, raffinose, and sucrose), four polyols (glycerol, mannitol, erythritol, and sorbitol), five amino acids (glycine, alanine, sarcosine, glycine betaine, and proline), and urea. Of the 16 solutes, 10 could be described in terms of a single parameter that is due to pure first-order effects (packing, hydration, or space limitation). The remaining six exhibit significant second-order effects (solute−solute interactions) and require two additional parameters, one typically identified with the volume occupied per solute molecule in the pure solute (crystal or liquid) and the other with a self-association constant. The activity coefficients of the osmolytes roughly display the rank order found with respect to their ability to stabilize proteins. These findings are discussed in terms of the physical principles that give rise to the activity coefficients.

The discipline of biochemistry has largely been built on the practice of isolating proteins and other macromolecules and studying their function(s) in dilute buffer saline solutions. The advantage of carrying out thermodynamic and kinetic measurements under such conditions is that, in dilute solution conditions, the thermodynamic activities of the reactants and products are very nearly equal to their concentrations, making it much easier to evaluate rate and equilibrium constants and develop an understanding of basic processes. It is common practice to assume that the results obtained in dilute solution under near thermodynamic ideal conditions are directly applicable to what takes place in the living cell from which the macromolecules were extracted. In stark contrast to these assumptions and practice, it is well-known that the aqueous solution within cells (the cytosol) is highly nonideal, containing highly concentrated total protein and other macromolecular components as well as significant concentrations of certain ions and small molecules. Clearly, there is a need to reconcile the application of biochemical results obtained under ideal conditions with the nonideal conditions existing in the cell (*1*−*4*).

The intracellular concentrations of some small molecules are particularly high in organisms that have adapted to water-stress conditions and in mammalian organs such as the kidney (*5*−*7*). These small molecules, known as organic osmolytes have the ability to counter the water stress and protect proteins and other cellular components against the denaturing conditions accompanying the water stress (*6*−*8*). The intracellular concentrations of organic osmolytes vary with the degree of water stress and can get quite high. An extreme example is that the urea concentration in kidney medulla cells of a water-stressed desert mouse has been reported as high as 5 M (*9*). The Record laboratory has been particularly active in studies involving nonideality in *Escherichia coli* (*10*). The point is that with high concentrations of low molecular weight solutes and extensive macromolecular crowding, intracellular solutions are thermodynamically nonideal, making it highly unlikely that rate and equilibrium constants determined in dilute solution *in vitro* will adequately apply to the physiological conditions that occur within the intracellular compartment. To bridge this divide between the general emphasis on performing experiments in dilute solution and the need to evaluate biologically important reactions occurring in physiologically relevant nonideal solution conditions, it will be necessary to develop a foundation for understanding the sources and nature of

† Supported by a training fellowship from the W. M. Keck Foundation to the Gulf Coast Consortia through the Keck Center for Computational and Structural Biology, NIGMS 49760 (to D.W.B.), NIGMS 37675 (to B.M.P.), Robert A. Welch Foundation Grants E-1028 (to B.M.P.) and H-1444 (to D.W.B.).

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10.1021/bi048681o CCC: $27.50 © 2004 American Chemical Society
Published on Web 10/21/2004

nonideal behavior in concentrated solutions of small molecules such as osmolytes and move in the direction of understanding more concentrated protein−osmolyte solutions. This need is exemplified by recent findings contrasting protein unfolding inside and outside of *E. coli*. As a function of the urea concentration, two different proteins have been forced to unfold *in vitro* as well as inside *E. coli* cells (*11*, *12*). The results show that unfolding of the proteins in *E. coli* cells is significantly more responsive to urea concentrations than it is *in vitro*. This implies that the nonideality of small molecules in solution can strongly influence folding within the cell and *in vitro* in different ways.

Our goal in this paper is to develop a foundation for understanding the sources and nature of nonideal behavior in concentrated aqueous solutions of naturally occurring osmolytes. Here, we consider two component solutions; future work will extend this to include three components. Our work shows how useful analytical expressions for activity coefficients can be obtained from the grand canonical partition function for the system. Depending on the concentration scale chosen, between one and three parameters are required to describe accurately the activity coefficients of 16 polyols, sugars, amino acids, and urea over the entire range of solubility (or as far as data are available). In a previous paper (*13*), we showed how this treatment also works for salt solutions. With nonionic osmolytes it is shown how the leading terms of the grand canonical partition function contribute to deviations from ideality as a result of what appears to be two physical phenomena, molecular packing and apparent oligomerization of solute molecules. The advantage of the approach is that it provides a highly accurate description of the effect of osmolyte concentration on activity coefficients over the complete range of solubility, with apparently only two physical parameters needed to account for the nonideal deviations.

Upon dealing with the sources of nonideal behavior, we are immediately faced with the problem of which concentration scale (or scales) to use in expressing the results. In pursuing the statistical mechanical approach provided here and elsewhere (*13*), it becomes clear that different concentration scales provide different and complementary information about the sources of nonideal solution behavior, as well as providing different perspectives and insight into molecular-based interpretations of concentrated solutions. Because certain concentration scales are so entrenched in the field and for experimental and theoretical reasons (*14*, *15*), it is both useful and necessary to provide a foundation for expressing the results of the statistical thermodynamic approach in terms of multiple concentration scales.

The molar[1] scale (mol/L) plays an especially prominent role both in biochemical experiments and physical chemical theory, as in the well-known Debye−Hückel (*16*) and McMillan−Mayer theories of solution (*17*). Also, it has been argued that for purposes of interpretation the molarity or number density is the most appropriate scale to use in describing activity coefficients in aqueous solution (*18*). Accordingly, the molar scale will be a prominent part of the work described. Although the molal scale (mol/kg) is easier to work with experimentally, the molar scale (mol/L) provides unique (volumetric) information about the system that cannot be obtained using other scales. Within the statistical thermodynamic framework, we show how to interconvert between molarity and molality without the need for solution density measurements.

We conclude with a discussion of the implications of this work regarding the effects of osmolytes on protein folding.

## THEORY: RELATION BETWEEN CONCENTRATIONS AND CHEMICAL ACTIVITIES

The properties of neutral solute molecules in aqueous solutions depend on their immediate local environment. We consider the solute molecules as being in some microscopic open system that is embedded in a large macroscopic reservoir, the sample. Such a subsystem freely exchanges material with the reservoir and is an *open* or *grand canonical* system. All thermodynamic information on such a system is contained in the grand canonical partition function (*19*). Because the partition function can be formulated in many different ways, the issue of whether it is possible to extract thermodynamic information with reasonable effort depends on the choice of a formulation that fits the given system, as well as on the choice of one of several alternative mathematical pathways. Hill (*14*) suggested a way to deal with these choices in the case of two-component solutions. Recently, we modified Hill's approach to describe molal activity coefficients (*13*). In the following sections, we shall extend this theory to activity coefficients in any concentration scale and, by way of the molar scale, introduce a volumetric description of the solution.

*Partition Function.* In the case at hand, we want to easily extract activity based relations for the solute. In addition, it is convenient to deal with an isobaric system; therefore, we choose to take the temperature, pressure, chemical potential of the nonvolatile solute, and the number of solvent molecules ($T$, $p$, $\mu$, and $N_w$) in describing the system. Because we choose the number of water molecules $N_w$ rather than the chemical potential of the water $\mu_w$ as an independent variable, strictly speaking, the system is semigrand canonical rather than grand canonical. The semigrand canonical partition $Z$ function is (*14*)

$$Z(T, p, \mu, N_w) = \exp\left(-\frac{N_w\mu_w}{RT}\right) = \sum_N Y_N(T, p, N, N_w)f^N \quad (1)$$

As usual, this may be written as an activity-weighted sum of canonical partition functions $Y_N$ of closed systems (canonical ensembles) containing a fixed number of $N$ solute particles (right side of eq 1). The absolute activity of a single solute molecule is $f = \exp(\mu/RT)$. After Hill (*14*), we shall treat the canonical $Y_N$ as functions or parameters that contain information on the average interactions between molecules in the solution. Alternatively, the entire partition function could be expressed in terms of interaction potentials or cluster integrals (*17*), but this involves a level of complexity not required here. Because our purpose is to provide an approach that is not only rigorous but also readily applicable, we proceed with the partition function $Z$ given in eq 1.

[1] Please note that a distinction between *molar* and *molal* is important, primarily at high concentrations. *Molar* is defined as the number of moles of solute per liter of solution, while *molal* is defined as the number of moles of solute per kilogram of solvent.

To illustrate the advantage of formulating the partition function in the manner given in eq 1, we recognize that the properties of the solvent are separated from the properties of the solute. On the right side of eq 1, only the solute occurs explicitly, although information on water is contained in the $Y_N$. The middle segment, exp $(-N_w\mu_w/RT)$, of eq 1 contains the solvent explicitly.

Before calculating activity coefficients, we reformulate $Z$ to facilitate the calculation by eliminating the contribution to the activity of the standard chemical potential $(Y_1/Y_0)$ (*13*, *14*). In general, this contribution is inaccessible by direct measurement. The absolute activity $f$ is replaced by the relative activity $a = fY_1/Y_0$ to yield

$$Z = Y_0\left(\sum_N \frac{a^N}{g_N}\right) = Y_0\left(1 + a + \frac{a^2}{g_2} + \frac{a^3}{g_3} + ...\right) \qquad (2)$$

where the interaction parameter $g_N$ is defined by

$$g_N = \frac{Y_1^N}{Y_N Y_0^{N-1}} \qquad (3)$$

In the limit of strong interactions, $g_N$ corresponds to an $N$-merization (dissociation) equilibrium constant. This definition of $g_N$ differs from that given previously (*14*, *13*), because it does not contain a scaling factor for the size of the system, as given by, e.g., the number of water molecules $N_w$. The reason for excluding the scale factor is that, because we shall change between different concentration scales, it is inconvenient to redefine the $g_N$ for each concentration scale and replace $N_w$ by different measures of the size suitable for the respective scale.

*Calculation of Particle Numbers.* The number of solute particles $N$ is obtained by a derivative with respect to the absolute activity $f$

$$\langle N\rangle = \left(\frac{\partial \ln Z}{\partial \ln f}\right)_{T,p,N_w} = \left(\frac{\partial \ln Z}{\partial \ln a}\right)_{T,p,N_w} = \frac{a + 2a^2/g_2 + 3a^3/g_3 + ...}{1 + a + a^2/g_2 + a^3/g_3 + ...} \qquad (4)$$

This procedure of calculating the particle number from the grand canonical partition function is identical to the well-known way of calculating the number of bound ligands from Wyman's binding polynomial (*20*).

Depending on the degree of nonideality of the solution, the number of terms in eq 4 that will be significant will vary. In dilute solution, only the first term in the numerator and denominator contributes to the average particle number $\langle N\rangle$, and as the solution becomes concentrated, more terms will be required. We shall take experimental data as a criterion for determining the minimum number of terms necessary to accurately describe the data. Instead of using this empirical criterion, we could alternatively describe the physical clusters of molecules in the solution. Hill (*21*) pointed out that a description in terms of physical clusters has two disadvantages. First, it does not converge as fast as the empirical approach. Second, the definition of physical clusters in a densely packed liquid is highly arbitrary, and under certain circumstances, these two ways of handling the partition function are not too different. In dilute solution, the empirical and the physical approaches are indistinguishable, whereas at high concentrations, one cannot expect the terms in the empirical approach to describe physical clusters.

*Calculation of Concentrations in Different Scales.* Choosing a concentration scale in describing the physical properties of a solution can have significant consequences. As will be shown, aqueous urea behaves nearly ideally if described in terms of the molar scale as judged by the fact that the molar activity coefficient remains near unity as the molar concentration of urea is varied. By contrast, the molal activity coefficient of urea changes markedly as the molal concentration of urea increases. The reason for the differences between molal and molar activity coefficients as a function of their respective molal and molar concentrations for any solute is due to the physical basis on which the scales are defined.

At low solute concentration, the molal and molar scales are indistinguishable, but in the high-concentration limit, the two scales differ considerably. Because molality is based on a defined mass of solvent (viz. 1 kg of solvent), solutes completely miscible with the solvent can in principle reach an infinite concentration. For example, as the water content (solvent) of a glycerol solution goes to zero, the molal concentration of glycerol becomes infinite. The same highly concentrated solution expressed in molar concentration terms will be a relatively small number (approximately 14 mol/L), determined by the density of the pure solute as the solvent component becomes vanishingly small.

What the molal scale has to offer is that it is easy to use and manipulate experimentally (masses can readily be measured more precisely than volumes), and as we shall see, its use in the statistical thermodynamic treatment yields information that can be associated with solvation properties. At elevated concentrations, the molarity scale reveals volumetric properties of the solution, connecting the volumetric properties of the pure solvent and pure solute through the density and partial molar volume for mixtures of the solute and solvent. The advantage of this scale is that it focuses on the solute and is the preferred scale to be used in dealing with phenomena in which the solute number density provides the most insight into molecular behavior involving the system.

The relationships between concentration scales and chemical activity can be derived using eq 4. Molality $(m)$ is the number $\langle N\rangle$ of solute particles per kilogram of (solvent) water molecules $g_w$

$$m = \frac{\langle N\rangle}{g_w} = \frac{\langle N\rangle}{1000N_w/M_w} = \frac{\langle N\rangle}{N_w/m_w} \qquad (5)$$

where $N_w$ is the number of water molecules in $g_w$, $M_w$ is the molecular weight of water, and $m_w = 1000/M_w$ is the molality of pure water. This leads to an expression that relates the molality $m$ to the activity $a$ using eq 4

$$m = \frac{1}{g_w}\frac{a + 2a^2/g_2 + 3a^3/g_3 + ...}{1 + a + a^2/g_2 + a^3/g_3 + ...} \qquad (6)$$

The expression of molarity requires calculation of the volume. Average volume can be derived from the partition function analogous to eq 4 for the particle number

$$\langle V\rangle = -\left(\frac{\partial \ln Z}{\partial\, p/RT}\right)_{T,f,N_w} = \frac{V_0 + V_1 a + V_2 a^2/g_2 + V_3 a^3/g_3 + ...}{1 + a + a^2/g_2 + a^3/g_3 + ...} \quad (7)$$

The expression for the molarity can therefore be calculated from eqs 4 and 7 to be

$$c = \frac{\langle N\rangle}{\langle V\rangle} = \frac{a + 2a^2/g_2 + 3a^3/g_3 + ...}{V_0 + V_1 a + V_2 a^2/g_2 + V_3 a^3/g_3 + ...} \quad (8)$$

The volumes $V_n$ are the apparent volumes occupied by what might be thought of as a cluster of $n$ solute molecules plus a possible hydration shell in solution, and $V_0$ is the volume occupied by the water in the absence of the solute.

*Calculation of Activity Coefficients: Strategy and General Procedure.* The activity coefficient is usually defined as the ratio of the measured activity over the activity expected if the solution behaves in an ideal manner. Hence, the value of the activity coefficient depends on how we define "ideal". Often a direct proportionality between concentration and activity defines an ideal solution, a relationship likely to occur in the dilute solution limit. Yet, as seen above, concentration scales can differ drastically from one another, especially at high solute concentrations.

One way of defining the ideal behavior $a_{id}$ of the relative activity is to take into account only the first term in the numerator and denominator in eq 4 for $\langle N\rangle$ (*22*). This is achieved in the limit of low activity $a \rightarrow 0$. As a result, from eq 6, the ideal relative activity in the molal scale is $a_{id,m}$

$$a_{id,m} = mg_w \quad (9)$$

and for the molar scale $a_{id,c}$

$$a_{id,c} = cV_0 \quad (10)$$

Given the definition of ideal behavior (eqs 9 and 10), we now turn to the nonideal behavior of the activity ($a \gg 0$). The activity coefficient can then be determined as depicted in Figure 1 for both first- and second-order effects. We consider this in steps. First, the average number of solute molecules $\langle N\rangle$ as a function of activity is calculated according to eq 4. This results in a plot $\langle N\rangle$ versus ln $a$ that looks like a binding curve. In practice, this first step will be routinely obtained in a suitable concentration scale, like molality (eq 6) or molarity (eq 8). Such a function would be inverted in a second step as seen in Figure 1A (—) together with the ideal behavior (- - -). The two relevant cases for this paper are first- and second-order behavior labeled 1 and 2 in Figure 1. In the final step, we consider the activity coefficient obtained by normalizing the activity with respect to the ideal behavior versus the solute particle number (concentration).

Because one purpose of this paper is to provide a rigorous but straightforward means of dealing with activity coefficients, we shall restrict the discussion to no higher than second-order terms for aqueous solutions. This ensures that the calculations are never more complicated than a quadratic equation. As previously shown (*13*), the resulting equations are already sufficient to describe accurately a large number of aqueous two-component systems, covering a concentration range up to and including the solubility limit.

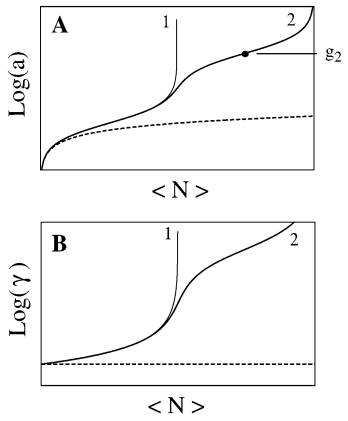


FIGURE 1: Activity and activity coefficients in the case of first-order (1) and second-order (2) behavior. (A) Relative activity $a$ as a function of the average particle number $\langle N\rangle$ is determined over a range of several orders of magnitude in a real solution (—) and an ideal dilute solution (- - -). (B) Activity is normalized to the ideal behavior. The extent to which the system tends to display second-order behavior strongly depends on the actual magnitude of the apparent dimerization constant $g_2$.

*First-Order Effects: Hydration, Packing Effects.* Calculation of the particle number $\langle N\rangle$ with subsequent conversion to a concentration scale is given by eq 6 for the molal scale and eq 8 for the molar scale. We now consider only first-order terms. The first-order expression for the molality (eq 6)

$$mg_w = \frac{a}{1 + a} \quad (11)$$

can be directly solved for the activity $a$

$$a = \frac{mg_w}{1 - mg_w} \quad (12)$$

Consider how this activity behaves as the concentration is varied from infinite dilution to high concentrations. At low concentrations, the denominator is essentially at unity, only the numerator is of importance, and the activity is directly proportional to the molality. As the concentration increases, the denominator approaches zero and the activity rapidly increases, finally to infinity.

We interpret this in terms of the number of water molecules $N_w$, which might be considered the hydration number of the solute molecule. In this view, the added solute claims hydration water that is no longer freely available to other solute molecules or the bulk solvent. As more solute is added to increase the concentration, the amount of free water decreases. Finally, hardly any free water is left for hydrating further solute molecules. Therefore, putting in more solute molecules, given the constraints of packing forces, requires more work as reflected in the hyperbolic increase in activity (labeled 1 in Figure 1).

The molal activity coefficient $\gamma_m$ is obtained by dividing the activity (eq 12) by the ideal behavior (eq 9)

$$\gamma_m = \frac{1}{1 - mg_w} \quad (13)$$

In the molar scale, the activity coefficient $\gamma_c$ is obtained along the same line of reasoning. The first-order version of eq 8 is solved for the activity and normalized using the ideal behavior (eq 10)

$$\gamma_c = \frac{1}{1 - cV_1} = \frac{1}{1 - c/c_1} \tag{14}$$

The parameter $V_1 = 1/c_1$ can be considered the effective volume occupied in the solution by the solute and its "associated" water of hydration. $c_1$ is the concentration that would be attained when all water is claimed by the solute. In practice, this concentration will never be reached because of the solubility limit.

*Second-Order Effects: Solute−Solute Interaction.* If we truncate at second order, eq 6 can be written as a simple quadratic equation

$$\frac{a^2}{g_2}(2 - mg_w) + a(1 - mg_w) - mg_w = 0 \tag{15}$$

that is solved in a straightforward manner to yield

$$a = \frac{g_2}{2}\frac{1 - mg_w}{2 - mg_w}\left[-1 + \sqrt{1 + \frac{4mg_w}{g_2}\frac{2 - mg_w}{(1 - mg_w)^2}}\right] \tag{16}$$

After division by the ideal molal activity (eq 9), the molal activity coefficient is obtained

$$\gamma_m = \frac{g_2/g_w}{2m}\frac{1 - mg_w}{2 - mg_w}\left[-1 + \sqrt{1 + \frac{4m}{g_2/g_w}\frac{2 - mg_w}{(1 - mg_w)^2}}\right] \tag{17}$$

Essentially the same procedure applied to the molarity scale leads from a rearranged second-order eq 8

$$\frac{a^2}{g_2}(2 - cV_2) + a(1 - cV_1) - cV_0 = 0 \tag{18}$$

to the activity

$$a = \frac{g_2}{2}\frac{1 - cV_1}{2 - cV_2}\left[-1 + \sqrt{1 + \frac{4cV_0}{g_2}\frac{2 - cV_2}{(1 - cV_1)^2}}\right] \tag{19}$$

Division by the ideal molar activity (eq 10) yields the molar activity coefficient

$$\gamma_c = \frac{g_{2,c}}{2c}\frac{1 - cV_1}{2 - cV_2}\left[-1 + \sqrt{1 + \frac{4c}{g_{2,c}}\frac{2 - cV_2}{(1 - cV_1)^2}}\right] \tag{20}$$

where for simplicity the definition $g_{2,c} = g_2/V_0$ is used.[2]

Figures 1 and 2 show two cases of activity coefficients resulting from second-order effects, the first one with weak solute−solute interaction and the second one with a stronger interaction. The former case of weak interaction shown in Figure 1 also includes pure first-order effects, manifested in the hyperbolic increase of the activity coefficient at low concentrations in B. This rapid increase is caused by the packing effects discussed in the previous section on first-order effects. As soon as second-order effects (solute−solute interactions) come into play, the activity coefficient dependence on concentration changes (see Figure 1B) and a second step in the "binding" curve is observed.

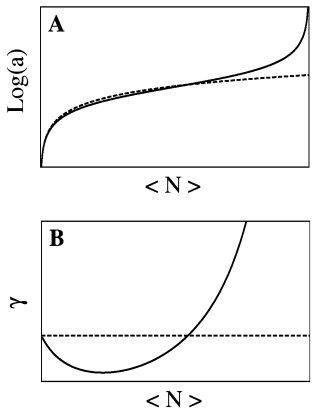


FIGURE 2: (A) Solution conditions are the same as in Figure 1 except $g_2$ is smaller (i.e., apparent affinity is larger), causing the middle portion of the plot to flatten (−). Ideal behavior is given by the dashed line. (B) Activity normalized to the ideal behavior giving the activity coefficient. Note the trend of $\gamma$ to values <1 because of dominant solute−solute contributions at low particle numbers; $\gamma$ swings up to values >1 as packing begins to dominate the solute−solute interaction effect.

If the solute−solute interaction is strong enough, it will mask the first-order packing effects as seen in Figure 2. In this case, the activity coefficient (Figure 2) drops below unity until second-order packing effects lead to a positive slope. It is important to realize that these effects are not always observable, because solute solubility typically limits the range in which activity coefficients can be observed. In some cases, like urea, this solute−solute interaction leads to a nearly complete counteraction of packing effects in the molal scale; i.e., the activity coefficient decreases well below unity with hardly any tendency for curving upward (*13*). As will be seen, such behavior is not expected in the molarity scale, because it has the density of the pure solute as a natural packing limit. From knowledge of the pure crystalline solute, one can calculate a maximal molar concentration. As discussed previously, in the molal scale, infinitely high concentrations are possible. This means that such a packing limit valid for the molar scale is absent in the molal scale. Only recruitment of water as hydration water can result in such apparent packing effects in the molal scale, and compounds with a very low apparent hydration like urea will not display any strong upward curvature (packing effects) of the activity coefficient in the molal scale.

This striking dependence of activity coefficients on the choice of the concentration scale arises from the characteristics of each scale. By their nature, the molar scale emphasizes volumetric properties of the solution components, while the molal scale emphasizes the impact of solvation (hydration) numbers.

*Conversion between Concentration Scales.* Usually activity-coefficient data are available in the literature in the molality scale. Therefore, a means for activity-coefficient conversion is needed, if one would like to have the activity

[2] Because $(1 - cV_1)^2$ in the denominator can go to zero, numerical difficulties in some fitting and plotting software may arise. For ease in computations, the equation should be modified by multiplying $(1 - cV_1)$ with the square brackets and the square root (see the Appendix).

coefficient in another scale, such as the molarity scale, typically used in biochemistry and biophysics. This task is made simple by using the theory developed in this paper. We shall show that molarity and molality can be rather accurately interconverted through knowledge of the densities of pure water and pure solute.

Conversion of molal into molar activity coefficients requires the multiplication of the molal activity coefficient by the molality ($mg_w$) and division by the molarity ($cV_0$). To readily accomplish this in the framework of the current theory, we seek a simple expression for the molarity $c$ in terms of the molality $m$. We start with eq 8, which gives the molarity as a function of activity in the simplest case (only first-order packing)

$$c = \frac{a}{V_0 + V_1 a} \tag{21}$$

The activity can also be expressed by the molality using the first-order version of eq 6

$$mg_w = \frac{a}{1 + a} \tag{22}$$

A combination of these two equations and eliminating the activity yield the desired relationship

$$c = \frac{mg_w}{V_0(1 - mg_w) + V_1 mg_w} \tag{23}$$

This is already an expression of the molarity $c$ in terms of molality $m$. It can be further simplified by noting that the mass of water $g_w = N_w/m_w$ divided by its volume in the absence of the solute $V_0$ is the density of pure water $\rho_w$. Moreover, if the given equation should describe the system from the infinite dilute aqueous solution up to the pure solute, the volume $V_1$ is the molar volume occupied by the pure solute. Then, $V_1$ is the inverse of the molarity $c_{max} = 1/V_1$ of the pure solute, and the equation for the molarity becomes

$$c = \frac{m\rho_w}{1 + m(\rho_w/c_{max} - g_w)} \tag{24}$$

If the crystalline solute does not contain any crystal water (a neat crystal), we must set the amount of hydration water $N_w = g_w m_w$ to zero to obtain the final expression for the molarity

$$c = \frac{m\rho_w}{1 + m\rho_w/c_{max}} \tag{25}$$

This first-order expression for the conversion of the molality to the molarity contains no adjustable parameter. It only depends on the density of the pure solute $\rho_{max}$ and the molar mass of the solute $M_r$ by way of the maximal molarity $c_{max} = \rho_{max}/M_r$. As will be seen below, employing this equation provides an essentially quantitative representation of the experimental relationship between molarity and molality concentration data for a number of aqueous two-component systems.

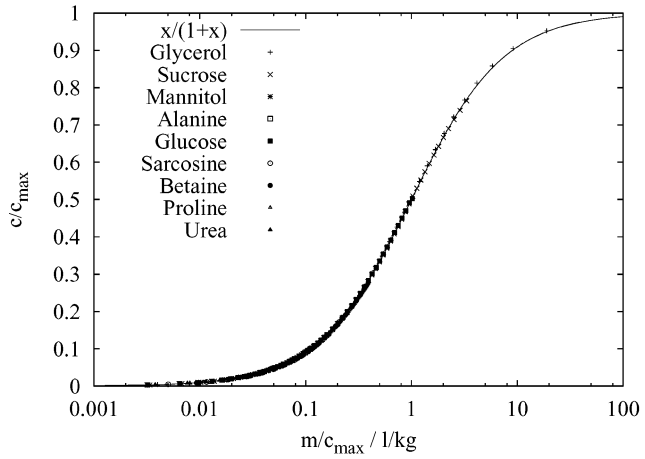


FIGURE 3: Molarity versus molality for a number of organic compounds in water. Both axes have been transformed according to eqs 25 and 26.

Selected data[3] are given in Figure 3. The sigmoidal line passing through the data is not a fit but is the one-parameter prediction based on eq 25 using anhydrous neat crystal (or liquid) density data from standard tables (*23*). To render all data in a form that allows easy comparison, both the molarity and molality have been divided by the $c_{max}$ characteristic for each compound. By this transformation, eq 25 gets a universal shape

$$f(x) = \frac{x}{1 + x} \tag{26}$$

where $f(x) = c/c_{max}$ and $x = m\rho_w/c_{max}$. There is reasonable quantitative agreement between eq 26 and the experimental data (Figure 3).

The curve is apparently universal and largely independent of the nature of the individual (uncharged) compounds. In addition, this agreement indicates that for the purpose of converting between molarity and molality our first-order expression of the partition function captures most of the relevant physics; i.e., the gross effect operative in solution is packing. We can also see that the molality is a good approximation for the molar activity $c/(1 - c/c_{max})$ in this special context. This is readily seen considering the ideal approximation for the density of the solution

$$\rho = \rho_0 + \frac{c}{c_{max}}(\rho_{max} - \rho_0) \tag{27}$$

which states that starting from the value at zero solute concentration $\rho_0$ the density $\rho$ changes in a linear fashion with increasing solute concentrations to the density of the pure solute $\rho_{max}$. This equation is inserted into the general rigorous relation between molarity and molality $m$ to yield

$$m = \frac{c}{\rho - cM/1000} = \frac{c}{\rho - c\rho_{max}/c_{max}} = \frac{c/\rho_0}{1 - c/c_{max}} \tag{28}$$

From this result, it is obvious that $m\rho_0$ equals the relative activity $a$ in the molarity scale obtained from a first-order

[3] Only data for uncharged (neutral and zwitterionic) organic molecules relevant for the current paper are shown. In the case of some salts, the hydration becomes important enough that $g_w$ in eq 24 can no longer be ignored.

Table 1: Molar Activity-Coefficient Parameters $g_{2,c}$, $V_1$, and $V_2$ Obtained from a Fit of the Given Experimental Data Using Eqs 14 and 20

| | $g_{2,c}$ (mol/L) | $1/V_1$ (mol/L) | $2/V_2$ (mol/L) | $c_{max}$ (mol/L) | highest $c$ (mol/L) | rmsd ($10^{-3}$) | data ref | density ref |
|---|---|---|---|---|---|---|---|---|
| glucose | | 6.28 | | 8.670 | 4 | 17.4 | *32*, *33* | *23* |
| xylose | | 7.6 | | 10.16 | 2.6 | 1.93 | *34* | *34* |
| maltose | | 3.135 | | 4.27[a] | 1.8 | 14.1 | *34* | *23* |
| mannose | | 7.04 | | 8.54 | 3.5 | 18.4 | *33* | *23*[b] |
| raffinose | | 1.523 | | 2.46[a] | 0.22 | 1.48 | *27* | *35*[b] |
| sucrose | 70.4 | 2.466 | $c_{max}$ | 4.617 | 2.6 | 8.05 | *29*, *26* | *23* |
| glycerol | 19 | 4.8 | $c_{max}$ | 13.69 | 7.1 | 5.77 | *29* | *23* |
| mannitol | | 7.35 | | 8.173 | 1.1 | 2.51 | *26* | *23* |
| erythritol | | 9.30 | | 11.88 | 3.8 | 1.59 | *28* | *23*[b] |
| sorbitol | | 6.475 | | 8.17 | 4.8 | 34.8 | *30*, *31*, *32* | *23*[b] |
| urea | 21.6 | 20.3 | $c_{max}$ | 22.03 | 10.1 | 1.23 | *37*, *36*, *29* | *23* |
| glycine | 3.765 | 3.260 | $c_{max}$ | 21.41 | 2.8 | 2.24 | *27*, *38* | *39* |
| alanine | | 14.40 | | 16.07 | 1.7 | 5.43 | *40*, *41* | *42* |
| proline | 120.5 | 5.38 | $c_{max}$ | 12.52 | 4.5 | 13.4 | *43* | *c* |
| sarcosine | | 8.68 | | 16.29 | 5.1 | 32.1 | *43* | *c* |
| glycine betaine | 16.88 | 1.97 | 4.94 | 10.72 | 3.4 | 13.8 | *43* | *c* |

[a] Density refers to hydrated crystalline solid. [b] Crystal density and eq 25 used. [c] Internal density data from Dr. Matthew Auton.

approximation to the partition function. We therefore see that the usage of the first-order expressions (eq 6 and 8) is essentially equivalent to approximating the density as a linear function of molarity.

## RESULTS: APPLICATION OF THE ACTIVITY-COEFFICIENT MODEL TO EXPERIMENTAL DATA

*Data Evaluation.* As experimental examples, we chose a number of compounds, as listed in Table 1. All isopiestic raw data were re-evaluated using as reference values the osmotic coefficients given by Archer for NaCl (*24*) and KCl (*25*) and by Robinson and Stokes for sucrose (*26*). The osmotic coefficients obtained were fit to a polynomial and converted to activity coefficients as described (*27*). For the exact conversion from molality to molarity, the concentration dependence of the densities of the aqueous solutions are required. They were taken from refs *23*, *34*, *39*, and *42* for half of the compounds. For the other half of the examples, density data were not readily available and were therefore either evaluated according to eq 25 or taken from unpublished data by Dr. Matthew Auton. The values for $c_{max}$ were taken from the literature (*23*, *35*). We shall separately publish density data for a number of common osmolytes along with a discussion of the volumetric behavior in light of eq 7.

*Sugars.* The molar activity coefficients of six sugars (glucose, xylose, maltose, mannose, sucrose, and raffinose) are shown in Figure 4. As a general trend, the saccharides are grouped according to their size. The activity coefficients of the three monosaccharides xylose, mannose, and glucose have the smallest slope, followed by the disaccharides maltose and sucrose. This trend continues with the much less soluble trisaccharide raffinose. In five of the six cases, first-order effects (eq 14) were sufficient to account for the data. The solute molecules generally occupy or have available a bit more space in the solution than in the crystal as judged by the parameters given in Table 1.

In most cases in which the data are consistent with first-order effects (eq 14), using the second-order eq 20 does not improve the fit. In the case of mannose and two other cases discussed below, eq 20 does yield a slightly better fit than eq 14 [root-mean-square deviation (rmsd) of $3.5 \times 10^{-3}$ in the case of mannose]. As long as the fit using the first-order eq 14 is still acceptable, we assume eq 14 to be the proper consistent description of the system. For comparison in these three cases, the second-order eq 20 is plotted as a dashed line together with the first-order eq 14 (—, Figures 4−6).

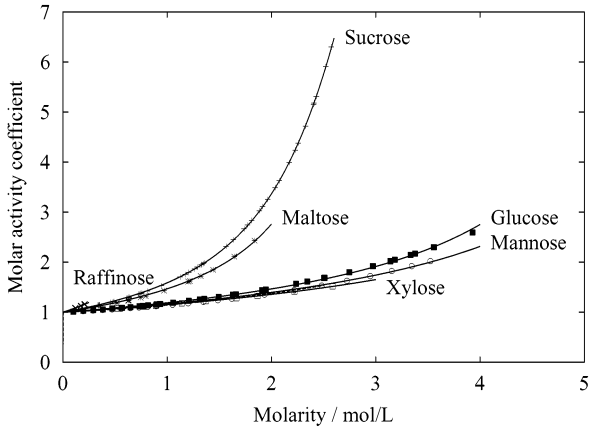


FIGURE 4: Molar activity coefficients of some sugars. The data were fitted to eq 20 in the case of sucrose and to eq 14 in all other cases. The resulting fit parameters are given in Table 1. The dashed line is explained in the text.

Sucrose is the only case among the six sugars in which second-order effects become apparent. If the volume associated with second-order effects $V_2$ (pairs of interacting molecules) is set to the volume of sucrose in the solid-state $2/c_m$ and kept constant, a good description of the data by eq 20 is obtained. This apparently reasonable assumption with regard to the nature of the second-order volume reduces the number of fitting parameters to two.

*Polyols and Urea.* The molar activity coefficients of four polyols (glycerol, mannitol, erythritol, and sorbitol) and urea are shown in Figure 5. Among the polyols, only glycerol clearly requires terms beyond the first order and has to be fitted using eq 20. Sorbitol can be fitted to the first order with little improvement going to the second order (rmsd is $25.6 \times 10^{-3}$ for the second-order fit), similar to the case of mannose. The other polyols exhibit only first-order effects and can be fitted with eq 14. As in the case of the sugars, possibly because of hydration, the volume $V_1$ occupied by the solutes in the solution is larger than the volume occupied in the pure substance. Also, the slope of the activity coefficient of the polyols increases with increasing size and

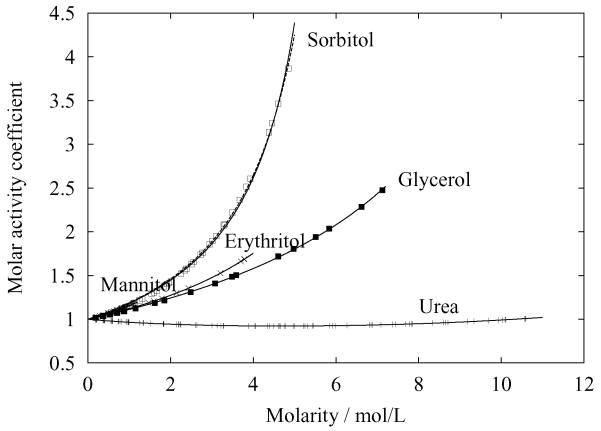


FIGURE 5: Molar activity coefficients of some polyols and urea. The data were fitted to eq 20 in the case of glycerol and urea and to eq 14 in all other cases. The resulting fit parameters are given in Table 1. The dashed line is explained in the text.

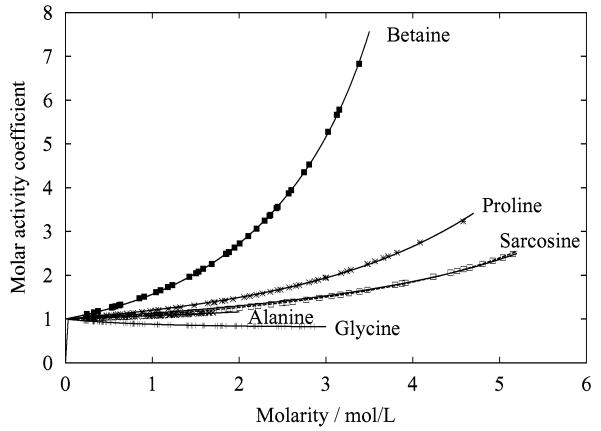


FIGURE 6: Molar activity coefficients of some amino acids. The data were fitted to eq 14 in the case of alanine and to eq 20 in all other cases. The resulting fit parameters are given in Table 1. The dashed line is explained in the text.

thus follows the same trend as the saccharides. They are sorted in the order: glycerol (three carbons), erythritol (four carbons), sorbitol, and mannitol (both six carbons).

Not only the activity coefficient of glycerol and sucrose but also that of urea involves second-order effects. In view of the large range of concentrations that is covered because of the good solubility of these compounds, this is not surprising. It is interesting, however, that, for these three cases, setting the second-order volume $V_2$ to the volume occupied by the solutes in their pure state is sufficient to account for the packing-related part of the second-order effects. This is consistent with an interpretation of this term as having its dominant contribution from contact association of pairs of molecules.

It is noteworthy that the molar activity coefficient of urea is always very close to unity. Therefore, in this special case, the usage of molarity instead of activity introduced hardly any error compared with doing so in the case of the other compounds discussed.

*Amino Acids.* The molar activity coefficients of five amino acids (glycine, alanine, proline, sarcosine, and glycine betaine) are shown in Figure 6. Among these amino acids, alanine is the only case in which the molar activity coefficients are strictly first-order. Sarcosine can be described by either first-order or first- and second-order terms (rmsd for the second order is $11.4 \times 10^{-3}$) and is therefore assumed to be represented by first-order terms. Glycine and proline show clear second-order solute−solute interaction effects. Glycine betaine, however, is special among all of the organic compounds discussed in this paper. In addition to being governed by second-order effects, the second-order volume $V_2$ is larger than the volume occupied by two glycine betaine molecules in the solid state. This indicates that at the highest concentrations the activity is already larger than the interaction parameter $g_{2,c}$. Therefore, glycine betaine hydration plays a much more significant role in its solution behavior than in the case of the other osmolytes, even at moderate solute concentrations.

Again, there is a rank order of activity-coefficient slopes. The addition of methyl groups to glycine progressively increases this slope as seen for the series: glycine, sarcosine, proline, and glycine betaine. The isomers sarcosine and alanine have very similar activity coefficients.

## DISCUSSION

The work presented here deals with the conversion between an intensive property (activity) and its conjugate extensive property (particle number, concentration) in a rigorous and straightforward manner. In thermodynamics, there are many pairs of conjugate properties: the activity relates to the concentration as the temperature relates to the heat (internal energy) or as the pressure relates to the volume. When this is put into perspective, although there may be temperature ranges in which heat and temperature are proportional, there is no general anticipation that they are always directly proportional (heat capacity varies with temperature). However, the physical chemistry of ideal dilute solutions with its simple direct proportionality between activity and concentration has been so successful that we must remind ourselves that the basis of this helpful but limited relationship is rooted in assumptions valid only at infinite dilution. Temperature is not an effective heat, pressure is not an effective volume, and activity is not an effective concentration.

In the next section, we shall discuss how the current approach enables a useful interpretation of chemical activity that must be considered because of failures in the classical interpretation of activity coefficients as effective concentrations.

*Interpretation of Activity Coefficients in Different Concentration Scales.* On the basis of the successful application of this approach to two-component systems here and elsewhere (*13*), it is useful to discuss how activity coefficients may be interpreted and the impact of the choice of a concentration scale in describing chemical activities quantitatively. First, on the basis of the observed behavior of urea, we give an instructional example that illustrates the importance of comparing activity-coefficient behavior using the molal and molar concentration scales. After that, the interpretation of the effects of the different scales are discussed in terms of the current statistical thermodynamic approach.

It will also become clear that the concept of activity as an effective concentration does not always result in a proper interpretation of the activity coefficients. Here is why. The expectation in terms of the classical point of view is that

solute hydration decreases the number of freely available water molecules in the solution (*45*), thereby effectively increasing the solute concentration or decreasing the effective water concentration (*46*, *47*). Such behavior, if dominant, will result in activity coefficients greater than the unity. On the other hand, an interaction between solute molecules may be considered an oligomerization. This would lead to an effective reduction of the solute concentration in the classical (colligative) point of view (*48*−*51*). Consequently, the classical view of the concept of activity as an effective concentration leads to the conclusion that an increase in the activity coefficient results from hydration, while a decrease in the activity coefficient arises from solute−solute interaction.

As shown by the specific example below, problems with this classical view occur immediately upon comparing molal and molar activity coefficients as functions of their respective concentration scales.

*Urea: Ideal Behavior in the Molarity Scale.* Urea is an important biological osmolyte, and because its intracellular concentration can achieve concentrations in the low molar range [e.g., in mammalian kidney (*5*, *9*)], it is important to understand its solution behavior. Unlike most solutes, the molar activity coefficient of urea is essentially at unity over the entire range of solubility (see Figure 5). Thus, on the molar scale, urea exhibits ideal behavior from the infinitely dilute solution range to 10 M. In contrast to the ideality shown by the molar scale for urea solutions, the activity coefficients based on the molal scale are not at unity over the concentration range and in fact are shown to decrease by a factor of 2 over the complete solubility range. From the classical (effective concentration) interpretation of activity-coefficient behavior, we are forced to confront the inconsistency that in terms of the molar scale urea is behaving ideally, whereas with the molal scale, urea−urea association appears to be the defining behavior. On the basis of the classical view, it is clear that the two scales provide conflicting interpretations.

To understand why molar and molal activity coefficients behave so differently, it is important to recognize that they probe different contributions to the solution behavior of urea and other solutes. A clue to these contributions comes from the partition function approach and is contained within the parameters of the analytical expressions that influence packing. Packing enters into the expressions for the molal activity coefficient $\gamma_m$ as an *apparent* hydration number $N_w$ or hydration mass $g_w$ (eqs 13 and 17), but in the case of urea solutions, the fact that $\gamma_m$ only decreases as a function of urea molality indicates that, if any hydration of urea ($N_w$) does take place, the packing contribution because of hydration is too weak to be significant within the range of urea solubility (*13*). Packing contributions arise in a different way with the molar scale. Inspection of the relevant terms (eqs 14 and 20) shows that with the molar activity coefficient $\gamma_c$ packing enters in the form of volumes. These volumes, $V_1$ and $V_2$, are composed of contributions from hydration and the van der Waals volume of the osmolyte molecule. Therefore, in contrast to molal activity coefficient $\gamma_m$, the molar activity coefficient $\gamma_c$ will always have packing contributions, because even if the volume of the hydration shell is negligible, the urea molecule itself physically contributes a volume to packing.

What then does the partition function approach say about hydration numbers in urea solution? First, on the basis of molal activity coefficients, the apparent hydration number $N_w$ or mass $g_w$ are too weak to come into effect in a significant way within the range of solubility (*13*). Second, as seen in Table 1, the fact that the first-order volume $V_1$ is barely different from the volume occupied in the dry urea crystal ($1/c_{max}$) and the fact that the second-order volume $V_2/2$ is indistinguishable from $1/c_{max}$, we have additional evidence that the effective hydration of urea in solution is quite low.

The analytical expressions derived from the partition function approach (and even the units) demonstrate that molal and molar activity coefficients are composed of different physical parameters. It should not be a surprise then that activity coefficients for the different concentration scales can behave quite differently as a function of the solute concentration. What is required of any approach is that the molecular interpretation of activity-coefficient behavior be internally consistent regardless of the concentration scale. Such internal consistency can be accommodated by the physical parameters in the partition function approach, but it is not adequately accommodated in the classical approach that involves direct application of effective concentration concepts.

A point that the partition function and classical approaches do have in common with respect to urea solutions is that both approaches conclude that urea is relatively unhydrated in solution. From a chemical point of view, hydrogen bonding between urea and water is expected to be highly probable. Some insight into this paradox comes from spectroscopic evidence that shows a small effect of urea hydration in solution (*53*) (on the order of 2 water molecules per urea molecule), but the population of more mobile water molecules increases in the presence of urea, while the relative amount of water in larger clusters decreases (*52*) (urea is a "structure breaker"). The opposing effects of some water being claimed by urea as a hydration shell, while other water molecules are freed from water clusters, allow for urea hydration (as expected on chemical grounds) in the local vicinity of urea molecules, while giving an overall average lack of specific or effective hydration thermodynamically. Thus, experimentally, the combination of the two effects results in an *apparent* lack of urea hydration.

As shown above, the unusual solution behavior of urea, with molar activity coefficients appearing to behave ideally, while molal activity coefficients behave in a nonideal manner, provides a basis for distinguishing between the classical and partition function approaches. With most aqueous solute systems, however, the classical and partition function approaches are not as easily distinguished because the molal and molar activity coefficients have similar trends as a function of the concentration. Consequently, hydration, hydration numbers, and solute−solute interaction by various definitions will have similar trends as well.

*First-Order Effects.* First-order behavior means that only terms up to the first order in the partition function are of significance. The only physical principle that comes into effect in the first-order case is solute-packing effects. As seen above, these packing effects are determined by the hydration number in the molal scale and by the effectively occupied space of the hydrated solute in the case of the molar scale. The example of urea shows that such effective hydration and

space limitation effects are *apparent* overall properties of the solution and do not necessarily reflect the actual specific hydration (first shell) of the solute molecule.

Here, we focus on the molar scale, and accordingly, the information on packing is obtained as apparent volumes of hydrated osmolytes. In the classical heuristic approach to the activity coefficients in aqueous solutions of organic solutes (*47*), hydration numbers are obtained. A direct comparison with the partition function approach is therefore only possible in the molal scale, which also yields (apparent) hydration numbers. Such comparisons were made in a previous publication (*13*). To summarize that work, hydration numbers reported using other (heuristic) approaches are of the same order as the apparent hydration numbers obtained with the partition function approach. Even if we ignore for the moment the conceptual problems associated with switching concentration scales, what makes the heuristic approaches less attractive is the virtually unlimited number of heuristic models that are capable of describing the behavior of a single solute. Sucrose is an example, where different heuristic models may contain more or fewer hydration sites or either exclude or include sucrose oligomerization (*54*). The advantage of the partition function approach is that it starts from first principles, thus avoiding arbitrary inclusions of properties that may or may not be present. The partition function is robust and is quite capable of handling systems more complex than encountered so far.

*Second-Order Effects.* First-order expressions involving a single parameter are often sufficient to account for the molar activity coefficients of many aqueous osmolyte solutions. The exceptions have required second-order expressions with a total of three parameters, namely, the first-order volume $V_1$, the second-order volume $V_2$, and the solute−solute interaction parameter $g_2$. We pointed out previously that in most cases (with glycine betaine being an exception) the curve fits were not sensitive to the magnitude of the second-order volume $V_2$. This is because the parameters $V_1$, $g_2$, and $V_2$ successively gain relevance with respect to the shape of the activity profile with increasing solute concentrations. This can be clearly seen in the case of well-separated first- and second-order effects as shown in Figure 1. The sigmoidal step in the left half of A is due to the first-order term, and the second sigmoid step in the right half is due to the second-order term. The position of the first step (in terms of ln $a$) is fixed, and it is defined by the ideal dilute solution behavior (- - -). The magnitude of the step (on the $\langle N \rangle$ axis) is defined by either the amount of hydration water $N_w$ or the effective size of the hydrated molecule $V_1$, depending on whether the molal or molar scale is used, respectively. Between the first- and second-order contributions (plateaus) in Figure 1A, the curve rapidly increases, which translates to a strong increase of the activity coefficient at intermediate $\langle N \rangle$ displayed in Figure 1B. The position (on the ln $a$ axis) of the plateau of the second sigmoidal step in Figure 1A is determined by the strength of the solute−solute interactions $g_2$, while the second-order volume $V_2$ again determines the magnitude of the step after conversion of $\langle N \rangle$ to molarity. Continuing with the activity coefficient displayed in Figure 1B, we therefore see that $g_2$ determines where the activity coefficient flattens at elevated concentrations $\langle N \rangle$. The volume $V_2$ comes into full effect only at much higher concentrations and is manifested by the second strong increase of the activity coefficient (partially shown) at the far right side of Figure 1B.

In the case of detectable solute−solute interaction as illustrated in Figure 2, a clear distinction between $V_1$- and $g_2$-related effects is not as straightforward as in the previously discussed case. However, the $V_2$-related rapid increase of the curve at the rightmost side at the highest concentrations $\langle N \rangle$ still takes place and occurs mostly outside the displayed ordinate range in Figure 2B. Only very few compounds will be soluble enough to have this $V_2$-related increase to come into effect within the range of solubility. For this reason, we cannot expect to obtain reliable information on $V_2$ from activity measurements alone; additional volumetric information is needed.

*Origin of the Observed Behavior of Activity Coefficients.* The examples given above show the necessity of developing a basis for interpreting activity-coefficient data. It is correct to think that the stronger a system is governed by solvation effects, the more the activity coefficient will have a tendency to curve upward. In addition, the stronger solute−solute attractive interactions become in solution, the more the activity coefficient will tend to go downward. In any case, as seen above, the actual functional dependence of the activity coefficient will depend strongly on the choice of a concentration scale.

It may well be that, in some binary system, the molal activity coefficient might drop below unity, while in the molar scale, it rises constantly. This kind of behavior is by no means contradictory. As shown earlier, it simply means that we observe different properties of the system when considering different concentration scales. The downward trend of the *molal* activity coefficient emphasizes that solute−solute interactions make important contributions within that concentration range, while a rising *molar* activity coefficient points out that the volume accessible to the solute molecules becomes limiting.

In the example of urea in which the molar activity coefficient $\gamma_c$ behaves near ideally, the inevitable effect of space limitations has to be compensated by solute−solute interactions to keep the molar activity coefficient $\gamma_c$ at unity. In addition to the decrease of the molal activity coefficient $\gamma_m$, because of solute−solute interactions, $\gamma_m$ can also decrease for other reasons at an extremely low water content. Increasing the molal concentration from nearly pure solute to absolutely pure solute does not change the solute activity but does cause the molality to become infinitely large. Therefore, a molal activity coefficient dropping to zero at extremely high concentrations is not indicative of solute−solute interactions, in contrast to a strong decrease observed at low to moderately high concentrations. The behavior at very high molal concentration is just a necessary consequence of the divergence of the molal scale for highly (or completely) miscible substances.

Depending on the system investigated, a certain concentration scale or approach might offer advantages over other scales. For example, in the current approach, using the molal scale begins with assuming a constant solvation number for the chemical species. In the case of macromolecules, a solvation number that remains constant clearly may not be a reasonable assumption. Different protein states, e.g., native versus denatured, can strongly differ with respect to their hydration. Using the molar scale solves this problem to some

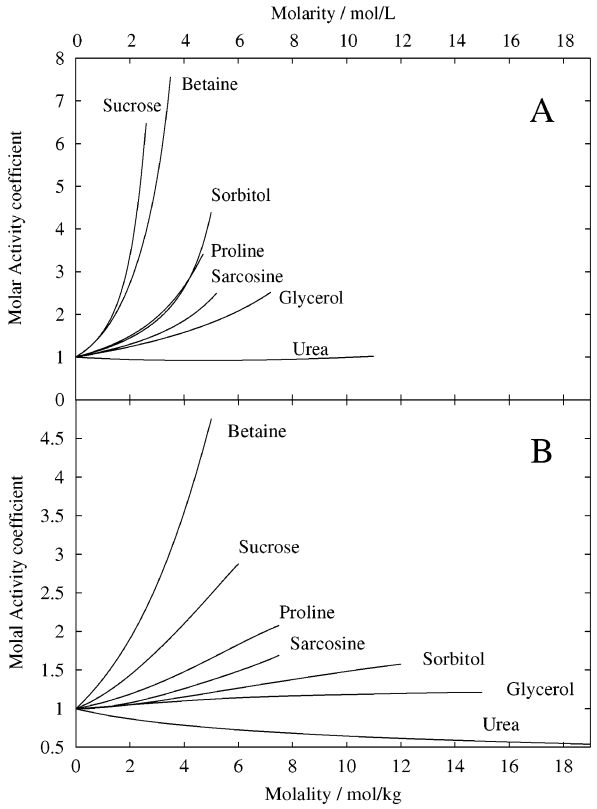


FIGURE 7: Comparison of molar (A) and molal (B) activity coefficients of several naturally occurring osmolytes.

extent, because a different volume can be assigned to each protein state. In a later publication, we shall show how to describe transitions involving large changes in solvation numbers.

*Impact on Protein Folding.* Naturally occurring osmolytes are known to influence the stability of proteins, with an important part of the overall effect being the relative preference of the peptide backbone for water or osmolyte (*44*). The change in Gibbs free energy upon transfer of the peptide backbone from a water to an osmolyte solution was recently measured, and a quantitative rank order of such transfer free energies was established (*55*). The preference of the peptide backbone for being hydrated decreases in favor of being solvated by osmolyte in the order TMAO > glycine betaine > sorbitol > sucrose > sarcosine > trehalose > proline > glycerol > urea. Activity-coefficient data are available for seven of the nine osmolytes. With a few exceptions, it turns out that the above-listed rank order of transfer free energies matches the rank order of molar activity coefficients when sorted by magnitude (see Figure 7A). The rank order with respect to molar activity coefficients is sucrose > glycine betaine > sorbitol > proline > sarcosine > glycerol > urea.

This rough correlation between the rank orders of activity-coefficient magnitude and transfer free energies of the peptide backbone (the main contribution to protein-folding efficacy) makes sense in view of the activity-coefficient model presented. An osmolyte like urea that favorably interacts with the peptide backbone of the protein (*55*) also has the tendency to interact with other urea molecules as judged by the down-curving molal activity coefficient (*13*) (Figure 7B). Both the destabilizing action on proteins and the decreasing molal activity-coefficient values correlate with the propensity of urea to interact with other organic matter (*56*), with modest *apparent* interaction with water (hydration). In the case of protein-stabilizing osmolytes, the strong increase of the molar activity coefficient is indicative of osmolyte hydration dominating the possibility of osmolyte−osmolyte interaction. This relative preference of osmolyte hydration over interaction with other organic matter is reflected in the osmophobic effect, i.e., the preferential hydration of the protein backbone compared with the backbone−osmolyte interaction (*44*). One might also argue that the strong hydration of stabilizing osmolytes, as interpreted from the activity-coefficient data, increases the effective size of the osmolyte, thereby contributing to preferential hydration by reason of steric exclusion (*57*, *58*). The relative deficit of cosolute close to the protein surface is a consequence of the preferential hydration of particular groups exposed on the protein surface (*44*, *55*). A third plausible effect is that, upon addition of an osmolyte that consumes a large amount of hydration water, the folding equilibrium is shifted toward the less hydrated native state. Protein hydration has long been thought to have a major impact on protein equilibria, even if direct interaction of the osmolyte with the protein cannot be neglected (*58*−*60* and references therein).

We shall provide a more detailed discussion of the impact of osmolyte activity coefficients on protein folding in a later paper on three-component systems. However, it is already clear at this point that, by considering the activity-coefficient behavior of osmolytes, it is possible to uncover underlying physical principles and better understand the basis of osmolyte action on protein-folding equilibria.

## CONCLUSIONS

Starting from basic principles, a statistical thermodynamic theory of solution is presented leading to easily applicable analytical expressions for solution nonideality that describe the behavior of molal and molar activity coefficients. The parameters that define the molal and molar activity-coefficient behaviors can be interpreted in terms of apparent solute oligomerization and apparent hydration, but it is also shown that the partition function approach provides internal consistency in explaining activity-coefficient behavior under experimental conditions, where the classical concept of activities as effective concentrations is found to be inadequate. We derive expressions for molarity and molality that lead to an essentially quantitative way of converting between these concentration scales without the need for experimental solution densities. The work provides a comparison between heuristic ways of interpreting solution behavior and the rigorous statistical thermodynamic approach presented here.

The nonideal solution effects in two-component systems demonstrate aspects of phenomena relevant to three-component systems that include proteins. Knowing the origin of the nonideal behavior of naturally occurring osmolytes is certain to have an impact on understanding protein folding and unfolding by these agents that can both protect and denature proteins.

## ACKNOWLEDGMENT

We thank Dr. Matthew Auton for providing density data on glycine betaine, sarcosine, and proline.

## APPENDIX A

*First-Order Activity Coefficients.* The first-order activity coefficients have a hyperbolic form in all concentration scales considered here. The molal activity coefficient is

$$\gamma_m = \frac{1}{1 - mg_w}$$

where $m$ is the molality and $g_w$ is the hydration number transformed into a mass.

In the molar scale $c$, the activity coefficient

$$\gamma_c = \frac{1}{1 - c/c_1}$$

contains the inverse molar volume $c_1$ of a solute-occupied subsystem.

The mole fraction scale $x_n$ activity coefficient

$$\gamma_{x_n} = \frac{1}{1 - x_n(1 + N_w)}$$

has as a crowding parameter, the total number of molecules $1 + N_w$ per subsystem, i.e., one solute molecule plus $N_w$ solvent molecules.

In the mass fraction scale $x_g$, the activity coefficient is

$$\gamma_{x_g} = \frac{1}{1 - x_g(1 + N_w M_{r,w}/M_r)}$$

where $M_{r,w}$ and $M_r$ are the molar masses of the solvent and solute, respectively.

*Second-Order Activity Coefficients.* Because the second-order equations for the activity coefficients in different concentration scales are all solutions of a quadratic equation, the general solution for the activity coefficient $\gamma_i$ in the concentration scale $i$ is

$$\gamma_i = \frac{-B_i + \sqrt{B_i^2 - 4A_iC_i}}{2A_iC_i}$$

where the functions $A_i$, $B_i$, and $C_i$ are slightly different for different scales ($i = m$, $c$, $x_n$, and $x_g$ for molal, molar, mole fraction, and mass fraction scales). Incidentally, $-C$ corresponds to the activity in an ideal dilute solution.

In the molal scale, we get

$$A_m = \frac{2 - mg_w}{g_2}, \quad B_m = 1 - mg_w, \quad C_m = -mg_w$$

where $m$ is the molality, $g_2$ is the pairwise interaction parameter, and $g_w$ is the hydration number transformed into a mass.

The calculations in the molar scale yielded

$$A_c = \frac{2 - c/c_2}{g_2}, \quad B_c = 1 - c/c_1, \quad C_c = -c/c_0$$

where $c$ is the molarity, $g_2$ is the pairwise interaction parameter, and $c_0$, $c_1$, and $c_2$ are the inverse molar volumes of subsystems containing 0, 1, or 2 solute molecules. Because, in the resulting equation, $g_2$ and $c_0$ always occur together, it is useful to combine them using $g_{2,c} = g_2c_0$ as shown above (eq 20).

The set of functions calculated in the case of the mole fraction scale is

$$A_{x_n} = \frac{2 - x_n(2 + N_w)}{g_2}, \quad B_{x_n} = 1 - x_n(1 + N_w), \quad C_{x_n} = -x_nN_w$$

Similarly, in the mass fraction scale, we get

$$A_{x_g} = \frac{2 - x_g(2 + N_w M_{r,w}/M_r)}{g_2}, \quad B_{x_g} = 1 - x_g(1 + N_w M_{r,w}/M_r), \quad C_{x_g} = -x_g N_w M_{r,w}/M_r$$

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BI048681O