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Daryl K. Eggers, San Jose State University
A. Y Payumo, San José State University
R. M Huijon, San José State University
D. D Mansfield, San José State University
L. M Belk, San José State University, et al.

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Changes in Apparent Molar Water Volume and DKP Solubility Yield Insights on the Hofmeister Effect

Alexander Y. Payumo, R. Michael Huijjon, Deauna D. Mansfield, Laurel M. Belk, Annie K. Bui, Anne E. Knight, and Daryl K. Eggers*

Department of Chemistry, San José State University, San José, California 95192-0101, United States

Supporting Information

ABSTRACT: This study examines the properties of a \(4 \times 2\) matrix of aqueous cations and anions at concentrations up to 8.0 M. The apparent molar water volume, as calculated by subtracting the mass and volume of the ions from the corresponding solution density, was found to exceed the molar volume of ice in many concentrated electrolyte solutions, underscoring the nonideal behavior of these systems. The solvent properties of water were also analyzed by measuring the solubility of diketopiperazine (DKP) in 2,000 M salt solutions prepared from the same ion combinations. Solution rankings for DKP solubility were found to parallel the Hofmeister series for both cations and anions, whereas molar water volume concurred with the cation series only. The results are discussed within the framework of a desolvation energy model that attributes solute-specific changes in equilibria to solute-dependent changes in the free energy of bulk water.

1. INTRODUCTION

The interior of a living cell is an extremely crowded environment where macromolecules occupy over 30% of the total solution volume.\(^{1,2}\) If one also considers the presence of smaller solutes and the fact that biomacromolecules are poly-electrolytes,\(^{3}\) it becomes evident that life’s vital reactions take place in a highly nonideal solution, in marked contrast to the dilute experimental conditions in which the properties of the individual reactants are typically characterized. In addition to excluded volume effects on reaction rates and equilibria due to macromolecular crowding,\(^{4,5}\) the innate properties of water may be altered in biological systems, although this issue is rarely addressed in the literature.\(^{6}\) New biophysical methods, such as in-cell NMR spectroscopy,\(^{7,8}\) have been developed for examining proteins in their natural environment, but such techniques may not be feasible or adequate for characterizing all biomolecules of interest, including the solvent.

To gain a better understanding of molecular activity in vivo, the properties of model compounds and model reactions may be determined in aqueous, nonideal solutions of defined composition. In the current work, solutions of concentrated electrolytes are examined to quantify two properties of water: the apparent molar water volume and the maximum solubility of diketopiperazine. Diketopiperazine (DKP), also referred to as cyclic diglycerol or glycine anhydride, is an amide-containing compound with the same bond connectivity as that found in the backbone of a protein. Electrolyte solutions are interesting for these studies because specific ions have been shown to influence the properties of biomolecules in a recurring order, known to biochemists as the Hofmeister series.\(^{9,10}\) Although it has been hypothesized often that ion-specific effects are mediated through changes in the properties of water, the underlying basis for the Hofmeister series is still highly debated,\(^ {11,14}\) and a single theory may not capture all nuances of this complicated biophysical problem. In vitro studies with nonideal solutions should facilitate a deeper understanding of water’s role in cell biology, and a thoughtful selection of electrolytes for such studies may also yield insights on the forces that drive the rankings of the Hofmeister series.

2. EXPERIMENTAL METHODS

Stock solutions of each salt were made at room temperature from ultrapure water (Millipore, Milli-Q system) and the following reagents: LiCl (99%, Fisher), KCl (99%, Sigma), CsCl (99.99%, Acros), N(CH\(_3\))\(_4\)Cl (97%, Aldrich), LiC\(_2\)H\(_3\)O\(_2\) (dihydrate, 98%, Acros), K\(_2\)H\(_2\)O\(_2\) (99%, Fisher), Cs\(_2\)H\(_2\)O\(_2\) (99.99%, Aldrich), N(CH\(_3\))\(_4\)C\(_2\)H\(_3\)O\(_2\) (hydrate, 95%, Acros), and LiClO\(_4\) (trihydrate, 99%, Acros). For solubility experiments, solutions were supplemented with 0.010 M Tris hydrochloride prepared from a 1.00 M stock buffer solution of pH 7.4 (Sigma, T2663). The final pH value of each salt solution was not adjusted. Diketopiperazine was obtained from Sigma (G7251).

Stock solutions were diluted with ultrapure water to obtain final electrolyte concentrations in the range of 0.2500–8.000 M, depending on the specific salt and its maximum solubility. All solutions were analyzed at atmospheric pressure and at four temperatures: \(T = 278.15, 298.15, 310.15,\) and 323.15 K. Solutions were maintained in capped vials at the desired air temperature for several hours in a closed incubator prior to density
measurements at the same temperature using a high-precision oscillating U-tube density meter (model DMA 5000, Anton Parr). This instrument has a rated uncertainty of $\pm 1 \times 10^{-6}$ g cm$^{-3}$ and a temperature uncertainty of $\pm 0.001$ degrees. The density meter was calibrated with air and ultrapure water following the manufacturer’s protocol. The apparent molar volume of each solution was calculated with the following equation:

$$\theta V_1 = \frac{M_i [1000 - C_i (4/3)\pi (r_1^3 + r_2^3)]N_A}{1000 \rho - M_2 C_2}$$  \hspace{0.5cm} (1)$$

where $\theta V_1$ is the apparent molar volume in units of cm$^3$ mol$^{-1}$, $r_1$ and $r_2$ are the Pauling radii of the cation and anion in centimeters, $N_A$ is Avogadro’s number, $C_i$ is the salt concentration in molarity, $\rho$ is the solution density in g cm$^{-3}$, $M_i$ is the molecular mass of water (18.015 g mol$^{-1}$), and $M_2$ is the formula mass of the anhydrous salt in g mol$^{-1}$. Values for the ionic radii were taken from Marcus$^{12}$ as listed here in Angstroms: Li$^+$ = 0.60; K$^+$ = 1.33; Cs$^+$ = 1.69; N(CH$_3$)$_4^+$ = 2.80; Cl$^-$ = 1.81; ClO$_4^-$ = 2.36; CH$_3$COO$^-$ = OAc$^-$ = 2.32. Diketopiperazine solubilities in 2.000 M salt solutions were determined by density analysis using the density meter described above. For a given experiment, 8 – 10 vials were prepared with 3.50 cm$^3$ of salt solution in each vial and increasing amounts of DKP. The mass range of DKP addition was selected such that three or more vials were below the saturation point and three or more vials were above the saturation point. The vials were placed in an incubator at the desired air temperature and stirred on a rotary mixing device at low speed (Barnstead International, Labquelle model 400110). Samples were allowed to equilibrate for 36 – 48 h before analysis. Each sample was loaded into the inlet port of the density meter through a syringe filter of 0.22 µm pore size (Millipore, SLGP033RB). DKP solubility was determined by plotting solution density versus milligrams of DKP added for each salt solution. The presaturation points were fitted to a linear equation of positive slope, the densities of the postsaturation points were averaged, and the intersection of the two regimes was interpreted as the saturation point. The concentration of DKP at the saturation point in units of grams of DKP per 100 g of water may be converted from milligrams of DKP added per cubic centimeter of initial solution volume using the following expression,

$$c_i^{\Phi} = \frac{10c_i^{\Phi}}{\rho_0 - (M_2 C_2 + M_2 C_3)/1000}$$  \hspace{0.5cm} (2)$$

where $c_i^{\Phi}$ is the solubility of DKP in solution i in units of g cm$^{-3}$, $c_i^{\Phi}$ is the solubility of DKP in units of mg cm$^{-3}$, $\rho_0$ is the original density of the electrolyte solution before addition of DKP, $M_2 C_2$ refers to the formula mass and concentration of the electrolyte, and $M_2 C_3$ refers to the formula mass (157.6 g mol$^{-1}$) and concentration (0.010 M) of the Tris buffer. The original density value is employed, as opposed to the density at saturation, because the total volume of solution used to calculate $c_i^{\Phi}$ and the precise concentration of electrolyte are known only before addition of DKP.

### 3. RESULTS AND DISCUSSION

In this study, a $4 \times 2$ matrix of salt combinations was examined: Li$^+$, K$^+$, Cs$^+$, and N(CH$_3$)$_4^+$ were paired with two anions, chloride and acetate (OAc$^-$). In addition, lithium perchlorate was added to the solution list to provide a third anion for testing. This salt was deemed most appropriate for comparison of the anions because lithium is one of few cations that permits the preparation of perchlorate solutions above 1.0 M concentration. The ions were selected to encompass a broad range of effects on the basis of their relative positions within the Hofmeister series. The densities of the nine electrolyte combinations were determined for a range of solute concentrations at atmospheric pressure and at four temperatures: $T = 278.15$, 298.15, 310.15, and 323.15 K (see Tables S1 – S3 of the Supporting Information).

#### 3.1. Apparent Molar Water Volume

The measured solution density values were converted to apparent molar water volumes, $\theta V_1$, by subtracting the known volume and mass of each ion in each solution (eq 1). This calculation, which treats the volume of the solute as a constant and allows the density of water to fluctuate, is a departure from the traditional approach that treats the density of water as a constant and attributes changes in solution volume to changes in the apparent molar volume of the solute, $\theta V_2$. Apparent molar solute volumes and their use in calculation of partial molar volumes, $\nabla V_1$ and $\nabla V_2$, are examined and discussed in the Supporting Information.

A sampling of $\theta V_1$ results for the $4 \times 2$ matrix is given in Figures 1 and 2. The apparent molar water volumes are presented as a function of salt concentration at one temperature, 310.15 K (Figure 1), and as a function of temperature at one concentration, 2.000 M (Figure 2). Interestingly, the apparent molar water volume of nearly all salt solutions in this study exceeds the molar volume of neat water at the same temperature, and in several solutions of high salt concentration, the apparent molar water volume exceeds the molar volume of ice, 19.65 cm$^3$ mol$^{-1}$ at 273.15 K (Figure 1).

With regard to the cations, a direct concordance was observed between the Hofmeister series and $\theta V_1$. For each temperature, concentration and anion pairing that was examined, the apparent molar water volume increased in the order Li$^+$ < K$^+$ < Cs$^+$ < N(CH$_3$)$_4^+$. This ranking corresponds to an increase in radius and a decrease in the effective surface charge density of each cation. For the anions, the results indicate that each chloride solution has a lower $\theta V_1$ value than the corresponding acetate solution when paired with the same cation (compare Figure 1). This outcome is consistent with the effective charge density of the two anions; that is, the ion of highest charge density (Cl$^-$) resulted in the lowest value of $\theta V_1$.

More insight into anion-specific effects is obtained by comparing the apparent molar water volume of solutions made from lithium salts of chloride, acetate, and perchlorate at 310.15 K (Figure 3). When the solutions are compared on a basis of equal ion numbers per unit volume, the solute-dependent value of $\theta V_1$ increases in the order Cl$^-$ < OAc$^-$ < ClO$_4^-$ at all concentrations. It is important to note that this result is not consistent with the Hofmeister rankings of anions; acetate and perchlorate are found at opposite ends of the anion series with chloride positioned near the center. Thus, the anion-induced changes in water structure that lead to changes in apparent molar water volume do not correlate with the Hofmeister effect. Others have also observed a lack of correlation between water structure and the Hofmeister series using pressure perturbation calorimetry$^{17}$ and nonlinear optical spectroscopy of Langmuir monolayers.$^{17}$

Except for the specific case of LiCl at 0.500 M, the value of $\theta V_1$ was found to be greater than the corresponding volume of pure water at the same temperature in all solutions. At first thought, this result might be alarming because many ions are known to
cause electrostriction of the solvent, yielding a molar volume less than neat water. It should be noted, however, that the phenomenon of electrostriction is applicable only to dilute solutions. As pointed out by Marcus, the average center-to-center ion spacing of a homogeneously dispersed solution of spherical ions may be approximated by $0.940c^{-1/3}$ nm, where $c$ is the salt concentration in molarity. After subtracting the radii of the anion and cation, there may be space for only a few water molecules in solutions above 1.0 M concentration. For example, in a solution of 6.0 M CsCl, for which the ionic diameters are 0.169 nm for Cs$^+$ and 0.181 nm for Cl$^-$, the available hydration space is $0.517 - (0.169 + 0.181)/2 = 0.342$ nm, corresponding to slightly more than one water molecule between ions. Thus, for most of the nonideal solutions reported here, water molecules are under the constant influence of one or more ions at any given instant in time. Under these conditions, the structural properties of water are expected to differ drastically from that observed in neat solution. Presumably, the tug-of-war between ions for waters of solvation contributes to the nonideal behavior of concentrated electrolyte solutions, as exemplified by the observation that $V_f$ may exceed the value of ice (Figure 1).

Figure 1. Apparent molar water volume at 310.15 K as a function of electrolyte concentration for (a) the chloride salts, and (b) the acetate salts. The solid horizontal line of each panel denotes the molar volume of neat water at 310.15 K, and the dashed horizontal line denotes the molar volume of ice at 273.15 K.

Figure 2. Apparent molar water volume in 2.000 M salt solutions as a function of temperature for (a) the chloride salts, and (b) the acetate salts. The bottom curve of each panel denotes the molar volume of neat water at the corresponding temperature, as calculated from a standard water density table.

Apparent molar water volumes are not commonly reported in the literature. Dougherty has calculated a reciprocally related parameter, the apparent water density, by subtracting the mass and volume of the electrolyte from the solution density. The Dougherty study includes CsCl in a graphical presentation of the data, from which one may estimate an apparent molar water density of 0.963 g·cm$^{-3}$ for 2.0 M CsCl at 298 K. Taking the reciprocal of this value and multiplying by the molecular mass of water yields an apparent molar water volume of 18.7 cm$^3$·mol$^{-1}$, in close agreement with the value of 18.65 cm$^3$·mol$^{-1}$ reported here (Table S1). The Dougherty analysis was limited to solutions up to 3.0 M concentration and employed ionic radii from crystal data that differ slightly from the radii used in the current work.

3.2. Diketopiperazine Solubility. Solubility experiments with DKP were performed for a control solution of low ionic strength (0.010 M Tris buffer) and for 2.000 M electrolyte solutions using density measurements to ascertain the saturation point. Initial work was completed at the physiological temperature of $T = 310.15$ K for all salts in the $4 \times 2$ matrix. In general, DKP solubility followed the rankings of the Hofmeister series of
cations: at \( T = 310.15 \) K, solubility decreased in the order \( \text{Li}^+ > \text{K}^+ \approx \text{Cs}^+ > \text{N}[(\text{CH}_3)_4]^{+} \) for both the chloride and acetate solutions (Figure 4). It is often noted in the literature that the cation series of Hofmeister effects is harder to discern than the anion series, but the cations in this study yield clear and reproducible changes in the solubility of DKP. A similar ranking of cations, in reverse order relative to highest DKP solubility, was deduced for solutions that enhance the structure of apomyoglobin confined to the pores of a silica glass matrix.\(^{21}\)

Solubility experiments were expanded to \( T = 278.15, 298.15, \) and 323.15 K for the control solution and for 2.000 M solutions of lithium chloride, lithium acetate, and lithium perchlorate. The DKP solubility results for the 2.000 M lithium solutions are summarized in Figure 5. For a given temperature, the solubility of DKP decreases in accord with the Hofmeister series of anions such that \( \text{ClO}_4^- \gg \text{Cl}^- \gg \text{water} > \text{OAc}^- \). The high solubility of DKP in lithium perchlorate solutions is consistent with the denaturing effect of perchlorate on protein structure. Because the amide units of the polypeptide backbone are normally buried in the core of a folded protein and not in contact with solvent, an increase in DKP solubility is analogous to the increase in exposure of the amide units of a protein in the unfolded state.

A solubility of 1.69 g per 100 g of \( \text{H}_2\text{O} \), as reported in Table S4 for DKP in water at 298.15 K, is in excellent agreement with a value of 1.68 g per 100 g of solvent, as obtained by density measurements and reported independently by the groups of Bolen\(^{15,22}\) and Lee.\(^{14,25}\) Most previous studies on DKP solubility have focused on urea or osmolyte solutions. The Lee group has measured DKP solubility in 2.0 M electrolyte solutions of \( \text{KBr}, \text{KCl}, \) and KOAc at 298.15 K.\(^{23}\) The DKP solubilities reported in the current work for KCl and KOAc solutions were carried out at 310.15 K, precluding a direct comparison, but it should be noted that the Lee group also found a correlation with the Hofmeister series of anions: DKP solubility decreased in the order \( \text{Br}^- > \text{Cl}^- > \text{OAc}^- \) for all concentrations of potassium salts up to 4.0 M concentration.\(^{23}\)

The solubility approach was also employed in the classical works of Robinson and Jencks, who tested acetyl tetraglycine ethyl ester as a model compound for the backbone of a protein.\(^{24,25}\) The rankings of salt effects on the solubility of the tetraglycine derivative are in agreement with the rankings reported here for DKP (Figures 4, 5, Table S4).\(^{14,15}\)

### 3.3. Relationship between Solubility and Desolvation Energy

Recently, our laboratory proposed an equation for relating solubility of a model compound in two or more solutions to the corresponding desolvation energies:\(^{26}\)

\[
RT \ln \left( \frac{c_A^\phi}{c_B^\phi} \right) = n \left( \Delta G_A^\text{H}_2\text{O}^\text{H} - \Delta G_B^\text{H}_2\text{O}^\text{H} \right)
\]

where \( c_i^\phi \) is the saturation concentration of the model compound (\( \Phi \)) in moles per mole of water, subscripts A and B denote the two solutions, \( n \) is the moles of water that solvate one molecule of \( \Phi \), and \( \Delta G_i^\text{H}_2\text{O}^\text{H} \) is the molar desolvation energy for \( \Phi \) in solution \( i \). The desolvation energy term is defined by the following relationship:

\[
\Delta G_i^\text{H}_2\text{O}^\text{H} = G_i^{\text{bulk}} - G_i^{\text{solv}}
\]

where \( G_i^{\text{bulk}} \) represents the average molar free energy of the bulk water in solution \( i \) and \( G_i^{\text{solv}} \) is the average molar free energy of the \( n \) water molecules in the solvation sphere of model compound \( \Phi \) that are displaced upon precipitation. The value of \( G_i^{\text{solv}} \) is a
function of the surface chemistry of the model compound, and
the value of $\gamma_1^{bulk}$ is a function of all solutes in solution $i$, including
secondary solutes that do not participate directly in the reaction
of interest. Because water molecules are in a dynamic equilib-
rium between multiple subpopulations of differing energy, as
defined by the chemical boundaries of all solutes and surfaces in
contact with the solvent, it seems reasonable to treat the
thermodynamics of bulk water as a number-weighted average of
all of the subpopulations. With this thermodynamic frame-
work in mind, the Hofmeister effect is expected to correlate with
changes in the average free energy of the bulk aqueous phase, and
it is not surprising that the measurement of a specific structure-
related property of water does not always follow the Hofmeister
series of ions. Reaction equilibria are dictated by changes in free
ergy, and therefore, it seems logical that the effects of electros-
ytes, and secondary solutes in general, are realized through their
effects on the free energy of bulk water.

In the context of the current work, an electrolyte that
increases the free energy of bulk water will result in a more-
positive desolvation energy, increase the solubility of DKP,
and destabilize protein structure. Conversely, an electrolyte
that decreases the free energy of bulk water will correspond to
a more-negative desolvation energy, decrease DKP solubility,
and enhance protein structure. Thus, on the basis of differ-
ences in DKP solubility, $N(CH_3)_4Cl$ and all acetate solutions
in this study should be stabilizers, LiCl should be a weak
destabilizer, $LiClO_4$ should be a strong destabilizer, and both
KCl and CsCl should have small or negligible effects on
protein structure at 310.15 K (Figures 4, 5). Further experiments
are required to obtain the solute-specific free energy terms
given in eqs 3 and 4.

## CONCLUSIONS

The apparent molar water volume was calculated for solutions
of four chloride salts, four acetate salts, and one perchlorate salt
at high molar concentrations. The results indicate that $\partial V_1/\partial c$
increases with increasing solute concentration and is greater in
magnitude than pure water at the same temperature for all
solutions above 1 M concentration. At the highest electrolyte
concentrations, $\partial V_1/\partial c$ exceeds the molar volume of ice for several
ion pairings, underscoring the nonideal behavior of these sys-
tems. The solution rankings for apparent molar water volume
were consistent with the cation series of Hofmeister ions but not
with the anion series, supporting the idea that the Hofmeister
effect is not a function of any specific structural feature of water
but, rather, follows a thermodynamic quantity, such as the change
in the Gibbs free energy of bulk water, that accompanies all
changes in water structure.

The solvent properties of the electrolyte solutions in this
study were compared using DKP as a model compound. The
highest solubility was found in lithium perchlorate solutions,
consistent with the protein-denaturing property of perchlo-
rate. For all cations and anions examined, the DKP solubility
rankings followed the Hofmeister effect. This result is ex-
pected if changes in solubility are governed by changes in
the free energy of bulk water and if changes in the free energy of
bulk water follow the Hofmeister series of ions. Further
characterization of water in nonideal solutions may lead to a
better understanding of hydration effects on reaction equili-
bria in biological systems, including binding equilibria and
protein folding.

## ASSOCIATED CONTENT

Supporting Information. Solution densities with corre-
sponding values of $\partial V_1/\partial c$ (Tables S1–S3), DKP solubility in 2 M
solutions (Table S4), and a critical analysis of partial molar
volumes from apparent molar volumes. This material is available
free of charge via the Internet at http://pubs.acs.org.

## AUTHOR INFORMATION

Corresponding Author

*Phone: 408-924-4960. Fax: 408-924-4945. E-mail: daryl.eggers@sjjsu.edu.

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