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# Protein interactions near crystallization: a microscopic approach to the Hofmeister series

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**Abstract.** The salting-out effect of simple electrolytes on lysozyme has been studied by measuring the second virial coefficient $B_2$ of the osmotic pressure as a function of salt concentration, and for different salts. The aim of this work has been to find a microscopic counterpart of the empirical Hofmeister series for the efficiency of cations and anions in inducing protein crystallization. The experimental results show that, for large enough ionic strengths, $B_2$ scales linearly with the salt concentration. This trend is common to a number of different monovalent salts, however with efficiency strongly dependent on the specific anion. Conversely, changing the cation does not appreciably affect $B_2$. The significance of these findings for the investigation of protein interactions near crystallization is discussed.

## 1. Salting out and the Hofmeister series

Proteins are generally crystallized by exploiting the so-called 'salting-out' effect, induced by the addition of a consistent amount of a simple electrolyte [1]. The salting-out effect strongly depends on the kind of electrolyte. The different relative effects of cations and anions on protein solubility particularly highlight this salt specificity. Indeed, for most proteins the degree of solubility is weakly dependent on the kind of cation, while it strongly depends on the kind of anion. In particular, the so-called 'Hofmeister series' sets a well defined empirical scale of efficiency for the different ions in precipitating proteins from solution. Besides its effects on protein solubility, the Hofmeister series plays a major role in setting the trend of many physical properties of electrolyte solutions, from the values of the surface tension and of the dielectric constant, to changes in the location of the demixing line of aqueous mixtures, to the critical micellar concentration of surfactant solutions in the presence of electrolytes [2]. However, in spite of its evident relevance in the study of aqueous solution, the Hofmeister series has not been given so far a clear microscopic explanation.

Most experimental studies of electrolyte addition to protein solutions have dealt with effects on the single-particle structure, such as unfolding, denaturation and changes of enzyme activity. This is by itself a subject of major biochemical interest, but, although 'chaotropic' effects on the conformational structure are strongly related to the Hofmeister series, they will not be discussed in this study. On the other hand, salt-specific effects on the thermodynamics of protein solutions have been mainly studied by determining changes of macroscopic properties like solubility. In terms of an equivalent one-component description of the system, where changes of the solvent composition are taken into account through an effective interparticle interaction potential, the equilibrium solubility line corresponds to the freezing line of a simple

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substance. Although this is a fundamental feature of the phase diagram, it is generally quite hard to quantitatively predict from a theoretical model. The purpose of this paper is to study how virial coefficients, which are more directly connected to interparticle interactions, are related, for salting out protein solutions, to the ordering set by the Hofmeister series.

Recent studies suggest that the phase behaviour of globular proteins in salting-out conditions closely conforms to what is expected for a suspension of hard particles interacting via a very short-range attractive potential [3, 4]. The short-range nature of the attractive part of the potential is responsible for the observed metastability of the fluid–fluid phase separation with respect to crystallization. Indeed, when the range of the potential becomes much shorter than the hard-sphere diameter, the fluid–fluid phase separation line 'sinks' within the fluid–solid coexistence region [5]. From a microscopic point of view therefore, the addition of electrolyte to protein solutions amounts to trigger the onset of effective interparticle attractions.

Following suggestions from [4], we have recently performed light scattering measurements well into the metastable region where crystal nucleation eventually takes place, showing that the osmotic compressibility of lysozyme solutions closely conforms up to very high concentrations to the theoretical expression for a system of 'sticky hard spheres' [6]. This simple model assumes an attractive part of the potential that is totally determined by a single adhesion parameter $\tau$ playing the role of an effective temperature. The $\tau$ parameter seems to be linearly related to the physical temperature, at least in the investigated temperature range. Similar conclusions have been previously reached by Rosenbaum and Zukoski [7] through light scattering measurements of the second virial coefficient.

Although the general features of the interaction potential are therefore clear, its origin is still to be elucidated. In this work, we will try to find out a better characterization of the salting-out potential, and in particular to find its dependence on the nature and concentration of the added electrolyte.

## 2. Experimental method and results

For low particle concentrations, the osmotic pressure of a particle solution can be expanded in a power series of the particle volume fraction $\Phi$ as: $\Pi = (k_B T / V_P)\Phi(1 + B_2\Phi)$, where $V_P$ is the particle volume. The virial coefficient $B_2$ is a pure number, which depends on the nature and strength of the interaction potential through:

$$B_2 = \frac{1}{2V_p} \int \left(1 - \mathrm{e}^{-U(r)/kT}\right) \mathrm{d}^3 r. \tag{1}$$

$B_2$ is positive for interactions dominated by repulsive terms, and negative for mainly attractive potentials. For spherical particles interacting only through excluded volume repulsion (hard spheres, HS) we simply have $B_2^{HS} = 4$. For suspension of small particles, $B_2$ can be directly extracted from a measurement of the intensity of the light scattered by the solution, which for dilute solutions is related to the particle concentration $c$ by:

$$\frac{c}{I_S} = \frac{1}{M}\left(1 + 2\frac{M B_2}{v_s} c\right) \tag{2}$$

where $M$ is the molecular weight and $v_s$ the specific volume.

We have used six-times recrystallized lysozyme obtained from Seikagaku, Japan (lot No E96301), dissolved in NaAcO, and extensively dialysed for a week against an NaAcO+HCl buffer at pH = 4.7. The buffer concentration was chosen to be 25 mM, a value sufficient to stabilize the pH within the experimental protein concentration range, but still low enough to give a negligible contribution to salting-out effects [10]. For each selected salt and ionic

strength, the most concentrated samples were prepared by mixing buffered lysozyme and salt solutions, with controlled pH = 4.7, at twice the required final concentrations. These samples had a protein concentration ranging from a typical value of 35 mg $ml^{-1}$, down to about 20 mg $ml^{-1}$ for those more 'critical' samples, where fast crystallization was prone to occur. Protein concentration was determined by absorption at 280 nm, and the particle volume fraction $\Phi$ was calculated by using the lysozyme specific volume $v_s = 0.71$ ml $g^{-1}$. The light scattering apparatus, which has been described elsewhere [7], was modified in order to reduce as much as possible the sample volume. Therefore, a special 25 $\mu$l volume flow-through cell (Hellma model 176.752), with an optical path of 1.5 mm, was used in connection with a close filling system.

Although quite a few measurements of virial coefficients in lysozyme solutions have been made in the recent past [7–12], the wide range of tested experimental conditions (different pHs, nature and concentration of the added electrolytes, temperature, lysozyme batches and preparation protocols) still render the existing set of data relatively sparse. In order to quantitatively study the salting-out effect, we have chosen to focus on two specific points.

- We have taken an extensive set of measurements of $B_2$ in the presence of NaCl in a range of ionic strength $I$ between 0.2 and 1.3 M, in order to establish the functional dependence of the salting-out effect on salt concentration. For lysozyme at pH = 4.7, in the presence of monovalent salts at concentration larger than about 0.2 M, the Debye–Hückel electrostatic contribution to interparticle interactions (see following discussion) is essentially negligible [9]. Beyond 1.3 M, nucleation of lysozyme crystal, even at protein concentration as low as 20 mg $ml^{-1}$, becomes so fast as to put a severe limit to the feasibility of scattering measurements. All measurements were performed at 25 °C. Figure 1 shows the experimental results for the virial coefficient as a function of added NaCl. Values for $B_2$ are rescaled to the one for hard spheres, defining $b_2 = \left(B_2^{HS} - B_2\right)/B_2^{HS}$, which is therefore positive whenever an attractive term is added to the excluded volume contribution. As can be seen, a linear fit, intercepting the concentration axis very close to the origin, reasonably describes the data in the whole ionic strength range. Results from [7] and [8], which were obtained in fairly similar conditions, are shown for comparison. Both sets are consistent with a linear dependence of $b_2$ on NaCl concentration for large ionic strength. Notice that virial coefficients obtained for *low* ionic strength [10] *do not* depend linearly on salt concentration. It is known that in salting-out conditions the logarithm of protein solubility decreases linearly with salt concentration [1]. Therefore, we expect a linear relation between $b_2$ and the logarithm of protein solubility $S$. The inset of figure 1, where we have plotted an extensive set of data from [12] for lysozyme in a wide range of experimental conditions (different salt concentration, pH, buffer composition, temperature) confirms that $\log(S)$ is roughly linearly related to the experimental value of $b_2$.
- In order to shed light on the Hofmeister series in terms of interparticle interactions, we have chosen to work only with monovalent salts, whose effects at the DH level of description are plain and indisputable. For cations, we have explored the alkali sequence. For anions, more complex ions like thiocyanate, which are known to have a dramatic effect on lysozyme solubility, have been considered. Figure 2 shows the results obtained for different potassium salts. A striking difference between the effects of different anions is evident: even at very modest concentration, thiocyanate and iodide induce much stronger attractive interactions than bromide and chloride, with nitrate playing an intermediate role. This scale of efficiency in inducing attractive interactions coincides with that one observed for solubility, where the order is *reversed* compared to the Hofmeister series for most proteins. Although the investigated ionic strength range is restricted compared to

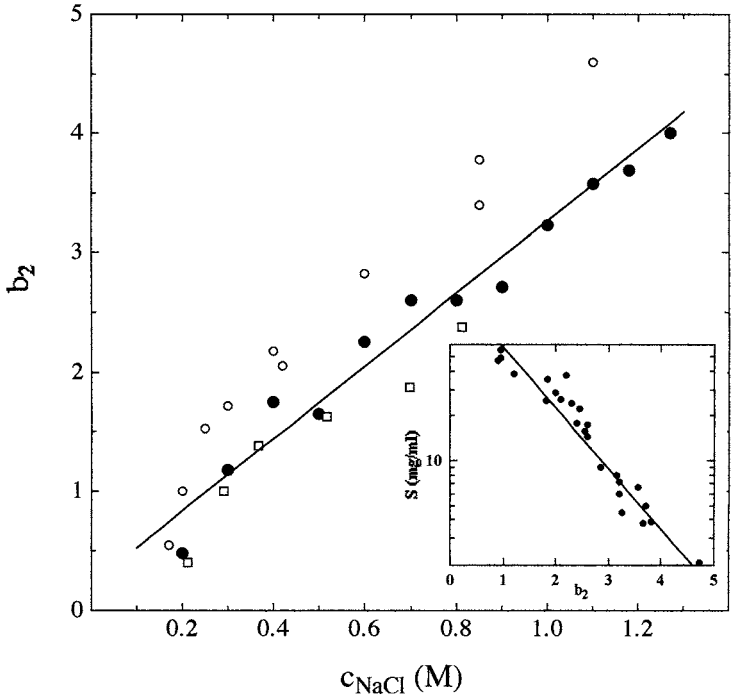


**Figure 1.** Dependence of the rescaled second virial coefficient $b_2$ on NaCl concentration for lysozyme solutions at pH = 4.7, $T = 25\,^{\circ}$C, fitted using a straight line. Open dots and squares represent data from [7] and [8]. The inset, showing data from [12], is discussed in the text.

the data for NaCl, an almost linear trend of $b_2$ with salt concentration can still be seen. The intercept with the $x$-axis is nearly equal for the different salts, and has an average value $c_0 \approx 0.06$ M (at variance with figure 1, this value is much better defined because of the steep slope for the strongly salting-out electrolytes). The inset of figure 2 shows therefore that all data can be superimposed on the data for a chosen salt, for instance KCl, by rescaling the salt concentration $c_s$ according to: $(c_{scal} - c_0) = \alpha(c_s - c_0)$, where the scaling factor $\alpha$ takes the values (1, 1.2, 4.7, 7.5, 8.6) for (KCl, KBr, $KNO_3$, KI, KSCN) respectively. Finally, figure 3 shows the results obtained for chlorides with different alkali cations. As a first observation, it is evident that salting-out effects are very weakly dependent on the nature of the cation. The residual effect shows however a curious 'inversion', since cations which are slightly more effective at low ionic strength become *less* effective at high ionic strength. This latter ordering is consistent with the Hofmeister scale for cations [2].

## 3. Discussion

It is customary to base a discussion of the phase behaviour of a suspension of charged particles in the colloidal size range on the classical DLVO theory for colloid stability. However, DLVO cannot properly account for salting-out effects [13], primarily because the electrostatic Debye–Hückel (DH) term in the DLVO potential depends only on the valence of the electrolyte, and therefore is not salt specific. Moreover, the amount of salt needed to induce protein crystal

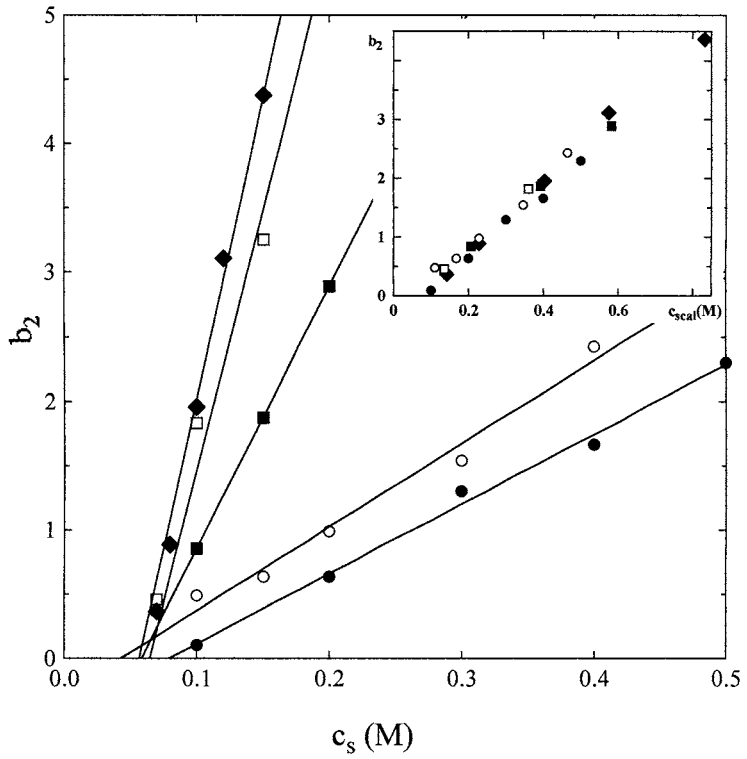


**Figure 2.** Rescaled virial coefficient for lysozyme solutions in presence of potassium salts with different anions, as a function of concentration of added electrolyte: KCl (full dots), KBr (open dots), $KNO_3$ (full squares), KI (open squares) and KSCN (diamonds). The inset shows the superposition of the data after rescaling salt concentration according to the text.

nucleation generally far exceeds what is needed for a severe screening of the DH repulsion, which should therefore play little role in typical crystallization conditions. Finally, DLVO models intrinsically metastable suspensions of lyophobic particles, and therefore cannot predict the observed coexistence of a fluid and an ordered crystal phase. Attempts to frame the salting-out interaction within DLVO theory have been based on salt-dependent dispersion forces, leading however to unrealistic values for the Hamaker constant [14], or on the introduction of salt-affected hydration layers. The only purely electrostatic theory, which goes beyond the DLVO level of description, and *does* predict salting-out effects at large enough electrolyte concentration is due to Kirkwood [15], who took into account the large dipole moment of proteins, showing that repulsive ion–protein interactions may be induced by image charges. Kirkwood theory cannot however account for the strong salt-specificity of salting out.

We tend to believe that salting out can only be accounted for by introducing an additional attractive term to the effective interparticle potential, brought in not by direct ion–protein interactions, but by an *indirect* mechanism, where an effective attraction stem from a favourable free energy balance in accommodating two particles in a close-by compared to a widely separated configuration. It is customary to split the free energy of insertion of a particle in a solvent into a 'hard' part, amounting to the work needed to create a cavity in the fluid, plus a 'soft' part, which takes into account specific interactions between the particle and the solvent molecules. Since proteins are much larger than solvent molecules, in a simple continuum

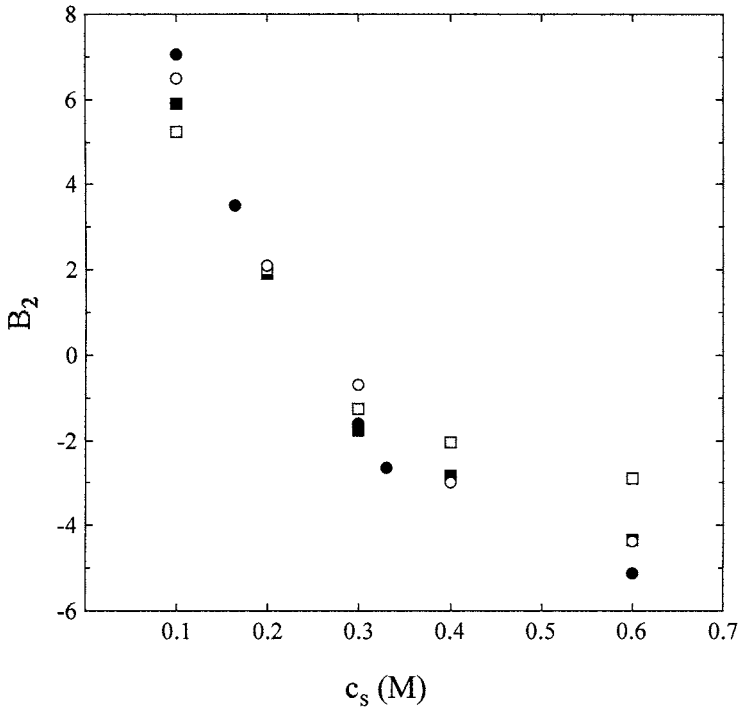


**Figure 3.** Virial coefficient of lysozyme solutions in presence of chorine salts with different cations, as a function of concentration of added electrolyte: LiCl (full dots), NaCl (open dots), KCl (full squares) and CsCl (open squares).

approximation the first term will be proportional to the surface tension of the solvent, which for electrolyte solution is directly related to the Hofmeister series. Surface tension data for a series of sodium salts [16] follow indeed not only the same order, but also roughly the same 'degree of effectiveness' expressed by the scaling coefficients for the virial coefficients we have found. We are presently working in order to derive more quantitative correlation. The effect on surface tension is supposed to be related to a depletion of salt ions at the interface, which in turn gives rise to a surface electric potential difference between water and air [2]. It is interesting to notice that the sign of the interfacial potential switches moving along the Hofmeister series for anions (being for instance large and positive for $SO_4^{2-}$, almost zero for $Cl^-$ and large and negative for $SCN^-$ salts). This suggests that the Hofmeister series will be followed in direct or reversed order depending only on the sign of the net protein charge. On the basis of the former observations, we believe that in order to derive a form for the salting-out potential it is important to work out a semiquantitative model for the work of cavity formation in an electrolyte solution. The basic questions to address at the level of statistical mechanics are therefore why the addition of electrolytes increases the surface tension of water, and moreover why anions are much more effective than cations in doing so.

It is interesting to notice that similar effective interactions, brought in through indirect mechanisms related to the exclusion of a solvent component from a given region, show up for colloidal suspension in presence of polymers or surfactants. High molecular-weight components like polymer, or spontaneous aggregates like surfactant micelles, lead indeed to depletion forces which do not require us to assume any specific interaction between polymer

coils and colloids. A depletion mechanism has been invoked by Mahadevan and Hall [17] to explain crystallization of proteins induced by polymers like polyethylene glycol (PEG). In particular, they predicted a linear decrease of the logarithm of protein solubility with PEG concentration in quantitative agreement with the experimental results. Since the hydrated size of an ion is not negligible compared to a typical protein radius, one could suppose that hydrated ions could play the role of depleting agent. Within this approach, salting out would be essentially an entropic effect. However, ion hydration is a 'fuzzy' concept, since no unambiguous definition of hydration layer has so far been given. Moreover, the linear dependence of $B_2$ on salt concentration cannot easily be understood using simple depletion ideas. Therefore, any possible interpretation of salting out as an entropy-driven effect should be based on a more specific model of aqueous salt solutions.

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