Mechanism of Protein Salting In and Salting Out by Divalent Cation Salts: Balance between Hydration and Salt Binding†

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ABSTRACT: The preferential interactions of proteins with solvent components were studied in concentrated aqueous solutions of the sulfate, acetate, and chloride salts of Mg^{2+}, Ba^{2+}, Ca^{2+}, Mn^{2+} and Ni^{2+} [except for $CaSO_4$, $BaSO_4$, $Mn(OAc)_2$, and $Ni(OAc)_2$], and results were compared with those of the Na^+ salts. It was found that, for all the salts, the preferential hydration increased in the order of $Cl^- < CH_3COO^- < SO_4^{2-}$ regardless of the cationic species used, in agreement with the anionic lyotropic series, and that the same parameter exhibited a tendency to increase in the order of Mn^{2+}, $Ni^{2+} < Ca^{2+}$, $Ba^{2+} < Mg^{2+} < Na^+$. The salting-out and stabilizing or salting-in and destabilizing effectivenesses of the salts were interpreted in terms of the observed preferential interactions. The surface tension increment of salts, which is a major factor responsible for the preferential interactions of the Na^+ salts, had no correlation with those of the divalent cation salts. It was shown that the binding of divalent cations to the proteins overcomes the salt exclusion due to the surface tension increase, leading to a decrease in the preferential hydration. In conformity with this mechanism, the preferential interaction of $MgCl_2$ was strongly pH dependent, because of the protein charge-dependent affinity of Mg^{2+} for proteins, while NaCl showed no pH dependence of the preferential interaction. The proposed mechanism was supported by a strong correlation between the preferential interaction results and the interaction of these salts with the model peptide compound acetyltetraglycine ethyl ester, described by Robinson and Jencks [Robinson, D. R., & Jencks, W. P. (1965) *J. Am. Chem. Soc. 87*, 2462–2470].

Salts affect in widely different manners the properties of macromolecules such as their stability, solubility, and biological activity (von Hippel & Schleich, 1969). At low concentrations, salts can stabilize proteins and other polyelectrolytes through nonspecific electrostatic interactions, dependent only on the ionic strength of the medium (Tanford, 1961). At high concentrations, however, salts exert specific effects on proteins which depend on the nature of the salt and its concentration, resulting in either the stabilization or denaturation of proteins, as well as in their salting in or salting out (either precipitation or crystallization). In a three-component system such as protein–water–salt, knowledge of the preferential interactions of the proteins with the solvent components can give an insight into the manner by which additives affect the solubility and stability of proteins. Those additives that show stabilizing or salting-out effects on macromolecules are characterized by a large preferential hydration of proteins (Timasheff et al., 1976; Pittz & Timasheff, 1978; Lee & Timasheff, 1981; Gekko & Timasheff, 1981; Lee & Lee, 1979, 1981; Arakawa & Timasheff, 1982a,b, 1983), whereas extensive binding of the additive to the proteins is frequently observed for those which have destabilizing or salting-in effects (Inoue & Timasheff, 1968; Timasheff & Inoue, 1968; Lee & Timasheff, 1974; Prakash et al., 1981). The former class includes 2-methyl-2,4-pentanediol (MPD), glycerol, and some sugars, salts, and amino acids. Such additives as guanidine hydrochloride (Gdn·HCl),[1] urea, some organic solvents, and certain salts belong to the second class.

In a recent paper (Arakawa & Timasheff, 1982a), we have reported that a good correlation exists between the effects of some salts and their preferential interactions with proteins. It has been shown for a series of Na^+ salts that the observed preferential hydration is the consequence primarily of the perturbation of the surface free energy change at the protein–solvent interface induced by the addition of the salts, in accordance with the cavity theory of Sinanoglu & Abdulnur (1964, 1965). No such correlation between preferential interactions and the surface tension increments in water were found for $MgSO_4$ and in particular for $MgCl_2$ (Arakawa & Timasheff, 1982a). Furthermore, the two Mg^{2+} salts were found to differ greatly from each other both in their preferential interactions with proteins and their effects on protein stability. A study of the preferential interactions of divalent cation salts with proteins was undertaken, therefore, to clarify these observations. As a corollary, the solubilities of some nonpolar amino acids in $MgCl_2$ and $MgSO_4$ solutions were measured to determine whether their interactions with salts are thermodynamically favorable or not, following a suggestion by Robinson & Jencks (1965a) that divalent cations in their hydrated form, e.g., $Mg^{2+}\cdot XH_2O$, bind to the peptide units through hydrogen bonds. The results of these studies and their analysis are reported in this paper.

Materials and Methods

Materials. Bovine serum albumin (BSA)[1] (89C-9300, 80F-9340) and β-lactoglobulin (β-LG) (119C-8015, 106C-8070) were purchased from Sigma. Lysozyme was from Sigma (57C-8025) and Worthington (30C671). Glycine and L-leucine were from Sigma, and L-tryptophan was from Schwarz/Mann. All salts were of reagent grade. $MgCl_2$ solutions were prepared by the dilution of a concentrated stock solution, made always from a freshly opened bottle because of the hygroscopicity of the salt. The pH of the salt solutions was adjusted with HCl or NaOH. In some cases, the salt solutions were prepared in 0.02 M glycine–HCl, phosphate, or glycine–NaOH buffers. The preferential interaction results

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[1] Abbreviations: BSA, bovine serum albumin; β-LG, β-lactoglobulin; Gdn·HCl, guanidine hydrochloride; ATGEE, acetyltetraglycine ethyl ester; OAc, acetate.

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obtained were consistent whatever method was used for the pH adjustment. Proteins were dissolved in water at high concentrations, dialyzed against distilled, deionized water, and lyophilized after passage through a sintered glass filter.

Protein Concentration Measurements. Protein concentrations were measured spectrophotometrically (Lee et al., 1979) by using absorbance values in dilute salt of 6.58 dL/(g cm) at 278 nm for BSA (Noelken & Timasheff, 1967), 27.4 at 281 nm for lysozyme (Roxby & Tanford, 1971), and 9.6 at 278 nm for β-LG (Townend et al., 1960). The absorptivities of the proteins in concentrated salts were determined as described previously (Arakawa & Timasheff, 1982b). These values were the following: for BSA, 6.60 in $MgCl_2$, 6.69 in $Ca(OAc)_2$ and $Ba(OAc)_2$, and 6.67 in $NiCl_2$, all at 1 M, and 6.63 in 2 M $MgCl_2$; for lysozyme, 27.8 in 1 M $MgCl_2$ and $MgSO_4$ and 27.7 in 1 M NaCl; for β-LG, 9.7 and 9.8 in 1 and 2 M $MgCl_2$, respectively. The wavelength used was the same as that in the dilute salt. When the absorptivities were not determined directly, they were estimated from the above values by assuming a linear relationship between absorptivity and salt concentration. Whenever the proteins were not fully soluble, their concentrations were measured in 6 M Gdn·HCl by using absorbance values of 6.25 at 278 nm for BSA, 26.9 at 281 nm for lysozyme, and 9.6 at 276 nm for β-LG.

Partial Specific Volume. Partial specific volumes were measured by using an Anton Paar DMA-02 precision densimeter, as described previously (Lee & Timasheff, 1974; Lee et al., 1979; Gekko & Timasheff, 1981; Arakawa & Timasheff, 1982b). The apparent specific volume, ϕ_{app}, was calculated by (Schachman, 1957; Kielly & Harrington, 1960; Casessa & Eisenberg, 1961, 1964)

$$\phi_{app} = (1/\rho_0)[1 - (\rho - \rho_0)/C] \quad (1)$$

where ρ_0 and ρ are the densities of the solvent and the protein solution, respectively, and C is the protein concentration in grams per milliliter. These measurements were carried out at conditions at which the molalities of solvent components and their chemical potentials were, in turn, kept identical in the protein solution and in the reference solvent. For the former measurements, proteins were dried in a vacuum oven at 40 °C over P_2O_5 for 2 days, and then the solvent was added to the dried proteins. For the latter measurements, the protein solutions were dialyzed against the solvent for 2 days at 20 °C with a change of fresh solvent. In cases where protein aggregation was observed (turbidity or precipitation), the protein solution was allowed to stand for about 30 min prior to measurement in a disposable syringe which had been sealed tightly with a plastic cap to avoid evaporation. It was then transferred directly to the densimeter cell, after discarding 1 mL from the syringe which might contain sedimented, large aggregates. This procedure was found to remove large aggregates that otherwise would sediment in the cell within the 15 min required for thermal equilibration and hence affect the density measurement. Density measurements were performed at 20 °C. The concentration dependence of ϕ_{app} was determined with five to six protein samples with concentrations varying between 5 and 30 mg/mL. The partial specific volumes of the proteins at constant chemical potential, $\phi_2'^0$, were obtained as the average of the ϕ_{app} values, since they showed little protein concentration dependence, whereas the values at constant molality, ϕ_2^0, were obtained by extrapolation of ϕ_{app} to zero protein concentration. Large errors in the measurements of ϕ_{app} due to protein aggregation would manifest themselves through a greater scatter in this plot than normally expected. In all cases, there was a good linearity in the ϕ_{app} vs. C plots.

The partial specific volumes of the salts were determined in similar manner. Salts that contained no water of crystallization were first dried in a vacuum oven at 70 °C over P_2O_5 and weighed into small test tubes. The solvent was then weighed into the same tubes, and they were sealed quickly with parafilm. The densities of the solvent and solutions were measured at 20 °C. The apparent specific volume, ϕ_{app}, was calculated with eq 1, where C, the salt concentration in grams per milliliter, was derived from

$$C = \rho W_{salt}/(W_{salt} + W_{solv})$$

where ρ is the density of the solution and W_{salt} and W_{solv} are the weights of the salt and solvent added to each test tube. Usually, six to eight salt solutions were prepared with concentrations of 20–100 mg/mL. A slight dependence of ϕ_{app} on C was observed in all cases, and $\bar{v}_3$ was obtained with high precision by extrapolation of ϕ_{app} to $C = 0$. The partial specific volumes of salts that contained water of crystallization were determined by the graphical method. These salts were dried in a vacuum oven at room temperature over P_2O_5. The dried salt and water were weighed into small test tubes, and the density measurements were performed as described above. It was assumed that the water of crystallization was not removed at these conditions, except for $MgSO_4 \cdot 7H_2O$ which is known to lose one water under relatively mild conditions. The weight fraction concentration of the salt, f_w, was calculated by

$$f_w = W_{salt}/(W_{total} + W_{H_2O}) \qquad W_{total} = W_{salt} + W_{cryst}$$

where W_{total} and W_{H_2O} are the amounts of salt and water weighed into the tubes and W_{salt} and W_{cryst} are the weights of the salt itself and the water of crystallization present in the salt sample. Samples were prepared so as to cover the weight fraction concentration of the salt at which the value of $\bar{v}_3$ was needed. The determined specific volume of the solution, i.e., the reciprocal of ρ, was plotted vs. f_w, and $\bar{v}_3$ was obtained graphically. Since the values of $\bar{v}_3$ needed were usually at $f_w = 0.1$–0.2, the graphical extrapolation to $f_w = 1$ led to some uncertainty of the results. The main source of error of this method is the long extrapolation while that of the weight method is the limit of sensitivity of the balance used.

Determination of Preferential Interactions. According to the notation of Scatchard (1946) and Stockmeyer (1950), component 1 is water, component 2 is protein, and component 3 is additive. The preferential interaction parameter of component 3 with protein, $(\partial g_3/\partial g_2)_{T,\mu_1,\mu_3}$, can be calculated from (Casessa & Eisenberg, 1961; Cohen & Eisenberg, 1968)

$$(\partial g_3/\partial g_2)_{T,\mu_1,\mu_3} = \rho_0(\phi_2^0 - \phi_2'^0)/(1 - \rho_0\bar{v}_3) \quad (2)$$

where g_i is the concentration of component i in grams per gram of water, μ_i is the chemical potential of component i, $\bar{v}_3$ is the partial specific volume of component 3, T is the thermodynamic temperature, and ϕ_2^0 and $\phi_2'^0$ are the partial specific volumes obtained at constant molality and constant chemical potential conditions, respectively, and extrapolated to zero protein concentration.[2] The same parameter in molal units, $(\partial m_3/\partial m_2)_{T,\mu_1,\mu_3}$, is

$$(\partial m_3/\partial m_2)_{T,\mu_1,\mu_3} = (M_2/M_3)(\partial g_3/\partial g_2)_{T,\mu_1,\mu_3} \quad (3)$$

where M_i and m_i are the molecular weight[3] and molality of

[2] The use of densimetry for the preferential interaction measurements in salt systems is particularly favored by the low values of $\bar{v}_3$ for salts which lead to great differences between ϕ_2^0 and $\phi_2'^0$.

[3] M_2 = 68 000 (BSA), M_2 = 36 800 (β-LG), and M_2 = 14 300 (lysozyme) were used throughout although β-LG dimer may dissociate at low pH.

component i, respectively. The preferential hydration parameter is obtained from (Timasheff & Kronman, 1969)

$$(\partial g_1/\partial g_2)_{T,\mu_1,\mu_3} = -(1/g_3)(\partial g_3/\partial g_2)_{T,\mu_1,\mu_3} \quad (4)$$

The preferential interaction parameter is a direct expression of the mutual perturbations of the chemical potentials of the protein and component 3 by each other, since, within a close approximation (Kirkwood & Goldberg, 1950; Casassa & Eisenberg, 1964)

$$(\partial m_3/\partial m_2)_{T,\mu_1,\mu_3} = -(\partial\mu_3/\partial m_2)_{T,P,m_3}/(\partial\mu_3/\partial m_3)_{T,P,m_2} = -(\partial\mu_2/\partial m_3)_{T,P,m_2}/(\partial\mu_3/\partial m_3)_{T,P,m_2} \quad (5)$$

where $\mu_i = \mu_i^0 + RT \ln a_i$, μ^0 and a_i are the standard chemical potential and activity of component i, respectively, P is pressure, and R is the universal gas constant. The self-interaction term $(\partial\mu_3/\partial m_3)_{T,P,m_2}$ was calculated from the molal mean ionic activity coefficient, $\gamma_\pm$. For 1:1 salts, $a_3 = m_3^2\gamma_\pm^2$, and for 2:1 and 1:2 salts, $a_3 = 4m_3^3\gamma_\pm^3$ (Robinson & Stokes, 1959). The preferential interaction parameter is related to the total bindings of component 3 and water to the protein by (Inoue & Timasheff, 1972)

$$(\partial g_3/\partial g_2)_{T,\mu_1,\mu_3} = A_3 - g_3A_1 \quad (6)$$

where A_i is the amount of component i in grams per gram of protein present in the protein domain within which the solvent properties are perturbed by protein molecules.

Solubility Measurements. The solubilities of glycine, L-leucine, and L-tryptophan in 1 or 2 M $MgCl_2$ and $MgSO_4$ solutions were measured densimetrically. Briefly, the dried amino acid and the solvent were weighed into several test tubes and left standing for more than 3 days at 20 °C, and after passage of the solutions through a Millipore filter, the densities of these samples were measured at 20 °C with the precision density meter. For all cases, a plot of the density, ρ, vs. the amount of amino acid in grams per gram of solvent showed that ρ first increases with a slight downward curvature and then becomes constant. This inflection point corresponds to the solubility of the amino acid. The constancy of ρ beyond the inflection point is also an indication of the purity of the amino acid sample. It was found that the solubilities of the amino acids in these solvents are high relative to those obtained in organic solvents.

Results

Preferential Interactions: Dependence on the Nature of the Ions. Preferential interaction measurements were carried out with the SO_4^{2-}, OAc^-, and Cl^- salts of Mg^{2+}, Ca^{2+}, Ba^{2+}, Mn^{2+}, and Ni^{2+}, except for $CaSO_4$ and $BaSO_4$ because of their low solubilities and for $Mn(OAc)_2$ and $Ni(OAc)_2$. The results using BSA as the protein are summarized in Table I. The value of ϕ_2^0 was found to be essentially independent of solvent conditions, although there was some scatter, probably reflecting differences between protein batches.[4] The values of $\bar{v}_3$ determined in this study are listed in Table II.[5] As reported previously (Arakawa & Timasheff, 1982a), the Na salts gave large negative values of $(\partial g_3/\partial g_2)_{T,\mu_1,\mu_3}$; i.e., BSA was preferentially hydrated in these salt systems. For the Mg^{2+}8 Ca^{2+}, and Ba^{2+} salts of Cl^- at pH 4.5–5.6, $(\partial g_3/\partial g_2)_{T,\mu_1,\mu_3}$ was not much different from zero within experimental error, indicating that the solvent compositions are more or less identical in the protein domain and in the bulk solvent. For $MnCl_2$ and $NiCl_2$, $(\partial g_3/\partial g_2)_{T,\mu_1,\mu_3}$ was significantly positive, indicating an excess of the salt in the protein domain over its concentration in the bulk solvent. Thus, when the salt is changed from NaCl to $MnCl_2$, this parameter changes from negative to positive, suggesting a large difference between Na^+ and the divalent cations and between the various divalent cations in their contribution to the overall preferential interaction. As is expected from $(\partial g_3/\partial g_2)_{T,\mu_1,\mu_3}$, the preferential hydration parameter, $(\partial g_1/\partial g_2)_{T,\mu_1,\mu_3}$, and the chemical potential change, $(\partial\mu_2/\partial m_3)_{T,P,m_2}$ were not significantly different from zero for the Mg^{2+}, Ca^{2+}, and Ba^{2+} salts of Cl^-. On the other hand, $(\partial\mu_2/\partial m_3)_{T,P,m_2}$ was significantly negative for the Mn^{2+} and Ni^{2+} salts; i.e., addition of these salts to the aqueous protein solution decreased the chemical potential of the protein and rendered the system thermodynamically more stable.

For all the divalent cation salts of OAc^- studied, $(\partial g_3/\partial g_2)_{T,\mu_1,\mu_3}$ was significantly negative, just as for NaOAc, indicating a deficiency of the salt in the protein domain relative to its concentration in the bulk. The resultant positive values of $(\partial g_1/\partial g_2)_{T,\mu_1,\mu_3}$ decreased from the Na^+ to the Ba^{2+} salt, in the same order as for the Cl^- salts. For NaOAc, $(\partial g_1/\partial g_2)_{T,\mu_1,\mu_3}$ was equal to the hydration value regarded normal for most proteins, 0.3 g/g (Bull & Breese, 1968; Kuntz, 1971; Kuntz & Kauzmann, 1974), while for the divalent cation salts it was lower, which suggests a penetration of the salt into the hydration layer of the protein. The positive value of $(\partial\mu_2/\partial m_3)_{T,P,m_2}$ for the acetate salts indicates an increase of the activity of the protein. For $Mg(OAc)_2$ and $Ca(OAc)_2$, this thermodynamic destabilization is comparable to that for NaOAc and somewhat higher than that for $Ba(OAc)_2$.

In the case of the SO_4^{2-} salts, $(\partial g_3/\partial g_2)_{T,\mu_1,\mu_3}$ was negative for all salts. The preferential hydration parameter increased in the order of $Ni^{2+} < Mn^{2+} < Mg^{2+} < Na^+$. For salts containing the same cationic species this parameter increased in the order of $Cl^- < OAc^- < SO_4^{2-}$, i.e., in a manner consistent with the Hofmeister series of anions for effectiveness as protein precipitants.

The preferential interactions of the Mg^{2+} salts were measured also with lysozyme. The results, shown in Table I, are qualitatively similar to those with BSA; i.e., $(\partial g_1/\partial g_2)_{T,\mu_1,\mu_3}$ increased in the same order for the anionic species. With this protein, $(\partial g_3/\partial g_2)_{T,\mu_1,\mu_3}$ for $MgCl_2$ was significantly negative. The somewhat higher values of $(\partial g_1/\partial g_2)_{T,\mu_1,\mu_3}$ for $Mg(OAc)_2$ and $MgSO_4$ with lysozyme than with BSA most probably reflect the difference between the surface areas per gram of the protein, in view of the known relationship between preferential hydration and the surface area of proteins (Timasheff et al., 1976; Lee & Timasheff, 1981; Arakawa & Timasheff, 1982a,b, 1983), as well as the differences in the chemical and physical properties of the proteins, e.g., their charged state, stability, and the nature and polarity of the protein surface.

The preferential interaction results are summarized schematically in Table III which is essentially a plot of the extent of preferential hydration as a function of the various ionic combinations. The correlation is good; i.e., $(\partial g_1/\partial g_2)_{T,\mu_1,\mu_3}$ is maximal in the corner corresponding to Na_2SO_4; it decreases going right and down in each row and column, respectively, and it is minimal with a negative value in the lower right hand corner corresponding to $NiCl_2$. The results are expressed somewhat more quantitatively in Figure 1, in which $(\partial g_1/$

[4] The partial specific volume may depend somewhat on the preparation. Since different batches of protein were used, the measurements of ϕ_2^0 and $\phi_2'^0$ were carried out always with the same batch.

[5] The accuracy of most of the $\bar{v}_3$ values is subject to considerable uncertainty due to the long extrapolation. This, however, does not affect the interaction results significantly when $\bar{v}_3$ is low, since $\bar{v}_3$ is used in the form $(1 - \rho_0\bar{v}_3)$. For example, for BSA in 1 M $MgSO_4$, an assumed value of 0.13 mL/g for $\bar{v}_3$ leads to $(\partial g_3/\partial g_2)_{T,\mu_1,\mu_3} = 0.0466$ g/g, whereas a value of 0.14 mL/g gives $(\partial g_3/\partial g_2)_{T,\mu_1,\mu_3} = 0.0471$ g/g; i.e., the difference in $(\partial g_3/\partial g_2)_{T,\mu_1,\mu_3}$ is only 1.3% although the $\bar{v}_3$ values differ by 7.7%.

Table I: Partial Specific Volumes and Preferential Interaction Parameters of BSA and Lysozyme in 1 M Salts

condition	ϕ_2^0 (mL/g)	$\phi_2'^0$ (mL/g)	$(\partial g_3/\partial g_2)_{T,\mu_1,\mu_3}$ (g/g)	$(\partial g_1/\partial g_2)_{T,\mu_1,\mu_3}$ (g/g)	$(\partial m_3/\partial m_2)_{T,\mu_1,\mu_3}$ (mol/mol)	$(\partial \mu_2/\partial m_3)_{T,P,m_2}$ [(cal/mol of protein)/ mol of salt]	$(\partial \mu_3/\partial m_3)_{T,P,m_2}$ [(cal/mol of salt)/ mol of salt]	$(\partial \sigma/\partial m_3)_{T,P,m_2}$	ratio
				BSA					
Na^+ salts									
NaCl, pH 5.6	0.735 ± 0.001	0.744 ± 0.001	−0.0145 ± 0.0032	0.243 ± 0.054	−16.8 ± 3.7	18300 ± 4000	1090	1.64	0.64
NaOAc, pH 5.6	0.735 ± 0.001	0.747 ± 0.001	−0.0270 ± 0.0043	0.312 ± 0.050	−22.4 ± 3.6	25200 ± 4100	1130		
Na_2SO_4, pH 5.6	0.735 ± 0.001	0.788 ± 0.001	−0.0739 ± 0.0028	0.524 ± 0.020	−35.4 ± 1.3	37200 ± 1400	1050	2.73	0.78
Mg^{2+} salts									
$MgCl_2$ pH 4.5	0.736 ± 0.001	0.739 ± 0.001	−0.0040 ± 0.0027	0.041 ± 0.027	−2.83 ± 1.89	7200 ± 4900	2540	3.16	0.13
$Mg(OAc)_2$, pH 5.6	0.734 ± 0.002	0.751 ± 0.001	−0.0277 ± 0.0048	0.180 ± 0.031	−13.2 ± 2.3	21700 ± 3800	1640		
$MgSO_4$, pH 4.5	0.734 ± 0.002	0.769	−0.0469 ± 0.0026	0.388 ± 0.022	−26.5 ± 1.5	17700 ± 1000	670	2.10	0.48
Ca^{2+} salts									
$CaCl_2$, pH 5.6	0.735 ± 0.001	0.731 ± 0.001	0.0052 ± 0.0025	−0.046 ± 0.021	3.20 ± 1.50	−7300 ± 3400	2290	3.66	−0.12
$Ca(OAc)_2$, pH 5.6	0.735 ± 0.002	0.749	−0.0292 ± 0.0042	0.170 ± 0.024	−12.6 ± 1.8	20300 ± 2900	1610		
Ba^{2+} salts									
$BaCl_2$, pH 4.5	0.737 ± 0.003	0.743 ± 0.002	−0.0081 ± 0.0068	0.038 ± 0.031	−2.65 ± 2.20	5000 ± 4100	1880		
$Ba(OAc)_2$, pH 5.6	0.735	0.751 ± 0.001	−0.0305 ± 0.0019	0.109 ± 0.007	−8.12 ± 0.51	11900 ± 700	1460		
Mn^{2+} salts									
$MnCl_2$, pH 4.5	0.737 ± 0.002	0.725 ± 0.001	0.0142 ± 0.0035	−0.110 ± 0.027	7.66 ± 1.89	−16900 ± 4200	2200		
$MnSO_4$, pH 4.5	0.736	0.755 ± 0.001	−0.0252 ± 0.0013	0.165 ± 0.008	−11.4 ± 3.8	7100 ± 2400	620		
Ni^{2+} salts									
$NiCl_2$, pH 4.5	0.740 ± 0.001	0.732 ± 0.001	0.0073 ± 0.0018	−0.056 ± 0.014	3.83 ± 0.95	−9400 ± 2300	2450		
$NiSO_4$, pH 4.5	0.737 ± 0.001	0.752 ± 0.001	−0.0169 ± 0.0023	0.109 ± 0.015	−7.42 ± 1.01	4200 ± 600	870		
				Lysozyme					
NaCl, pH 4.5	0.707 ± 0.002	0.723 ± 0.002	−0.0253 ± 0.0063	0.424 ± 0.106	−6.20 ± 1.54	6800 ± 1700	1090	1.64	0.68
$MgCl_2$, pH 4.5	0.712	0.721 ± 0.001	−0.0119 ± 0.0013	0.122 ± 0.013	−1.79 ± 0.19	4500 ± 500	2540	3.16	0.24
$Mg(OAc)_2$, pH 5.6	0.708 ± 0.001	0.733 ± 0.002	−0.0405 ± 0.0049	0.263 ± 0.032	−4.07 ± 0.49	6700 ± 800	1640		
$MgSO_4$, pH 4.5	0.710 ± 0.002	0.748 ± 0.001	−0.0498 ± 0.0039	0.412 ± 0.033	−5.92 ± 0.46	4000 ± 300	670	2.10	0.31

Table II: Partial Specific Volumes of Salts

cation	anion at 1 M (mL/g) Cl^-	OAc^-	SO_4^{2-}
Mg^{2+}	0.176	0.313	0.136
Ca^{2+}	0.216[a]	0.440	
Ba^{2+}	0.110	0.325	
Mn^{2+}	0.062		0.126
Ni^{2+}	−0.2		−0.021
Na^+	0.331	0.516	0.208

$MgCl_2$				
concentration (M)	0.5	1.0	1.5	2.0
$\bar{v}_3$ (mL/g)	0.150	0.176	0.195	0.200

[a] Dunn (1966).

Table III: Schematic Expression of the Preferential Interaction Parameter for Various Combinations of Cationic and Anionic Species[a]

cation	anion SO_4^{2-}	CH_2COO^-	Cl^-
Na^+	+++++	+++	++
			0000
Mg^{2+}	+++	++	+−[b]
	0000	000	0
Ca^{2+}		++	−+[c]
Ba^{2+}		+	+−[b]
Mn^{2+}	++		−
Ni^{2+}	+		−

[a] Results for BSA [(+) and (−)] and lysozyme (0). The number of marks [(0) or (+) and (−)] corresponds to 0.1 g/g in $(\partial g_1/\partial g_2)_{T,\mu_1,\mu_3}$. [b] $0 < (\partial g_1/\partial g_2) < 0.05$. [c] $-0.05 < (\partial g_1/\partial g_2) < 0$.

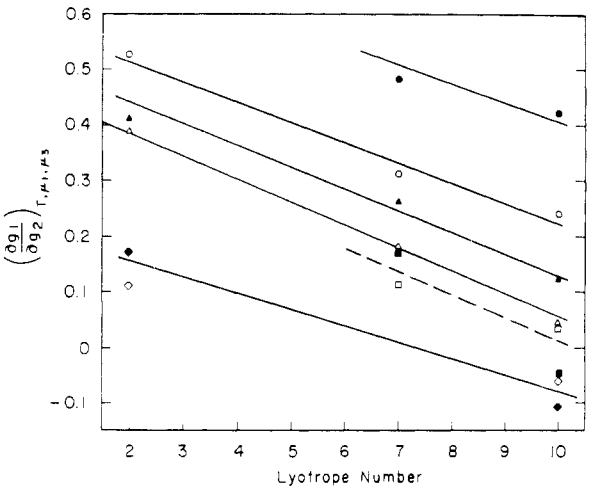

FIGURE 1: Correlation between $(\partial g_1/\partial g_2)_{T,\mu_1,\mu_3}$ of BSA and the lyotropic number for anions. Na^+ salts (○), Mg^{2+} salts (△), Ca^{2+} salts (■), Ba^{2+} salts (□), Mn^{2+} salts (♦), and Ni^{2+} salts (◊). The results for lysozyme are also given: Na^+ salts (●) and Mg^{2+} salts (▲).

$\partial g_2)_{T,\mu_1,\mu_3}$ is plotted as a function of the lyotropic index for anions (Voet, 1937). Although there is some scatter, it is possible to draw straight lines through the points of each cationic species, grouping together Ba^{2+} and Ca^{2+} salts and Ni^{2+} and Mn^{2+} salts. In this analysis as well, the contribution of the cationic species to the protein preferential hydration increases in the order $Ni^{2+} \leq Mn^{2+} < Ba^{2+} \leq Ca^{2+} < Mg^{2+} < Na^+$, regardless of the anionic species. The nearly parallel lines indicate that the protein preferential hydration increases by nearly identical increments in the order $Cl^- < OAc^- < SO_4^{2-}$, independently of the nature of the cations and of the proteins. This permits one to extend to divalent cation salts the relation between the preferential hydration and the salting-out effectiveness of anions which had been proposed for the Na salts.

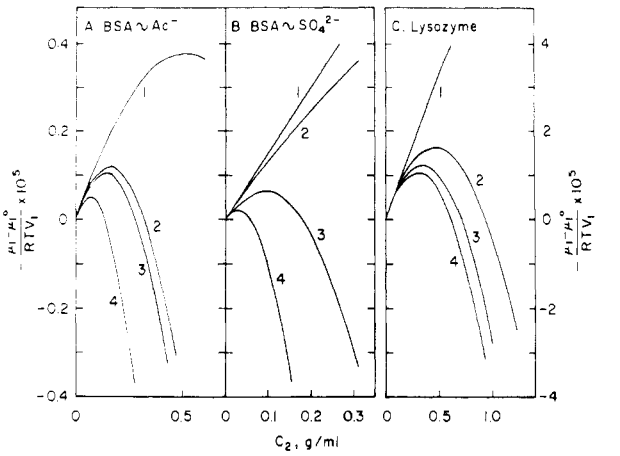

FIGURE 2: Phase isotherms for BSA in OAc^- salts (A) and in SO_4^{2-} salts (B) and for lysozyme in Mg^{2+} salts (C). (A) 1, Ba^{2+} (1.02); 2, Ca^{2+} (0.32); 3, Mg^{2+} (0.28); 4, Na^+ (0.14). (B) 1, Ni^{2+} (35); 2, Mn^{2+} (1.5); 3, Mg^{2+} (0.18); 4, Na^+ (0.06). (C) 1, $MgCl_2$ (7.0); 2, $MgSO_4$ (0.94); 3, $Mg(OAc)_2$ (0.71); 4, NaCl (0.62). Values given in parentheses are the protein concentrations in grams per milliliter at $\mu_1 - \mu_1^0 = 0$.

Salting-Out Effectiveness. The increase in the chemical potential of proteins upon addition of the divalent cation salts of OAc^- and SO_4^{2-} should decrease protein solubility. The extent of this decrease was estimated from the phase isotherm (Timasheff et al., 1976; Pittz & Timasheff, 1978)

$$(\mu_1 - \mu_1^0)/(RTV_1) = -(C_2/M_2)[1 + [V_m/(2RTM_2)]C_2[(\partial\mu_2^{(e)}/\partial m_2)_{T,P,m_3} + (\partial m_3/\partial m_2)_{T,\mu_1,\mu_3}(\partial\mu_2/\partial m_3)_{T,P,m_2}] + O(C_2^2)] \quad (7)$$

where V_1 is the molar volume of water, V_m is the volume of solution containing 1 kg of principal solvent, C_2 is the protein concentration in grams per milliliter, and $\mu_2^{(e)}$ is the excess chemical potential of the protein. Negative values of $\mu_1 - \mu_1^0$ indicate a stable solution, and positive values mean instability of the system, resulting in phase separation. This phase isotherm was calculated for BSA and lysozyme with the simplification that $(\partial\mu_2^{(e)}/\partial m_2)_{T,P,m_3}$ is due solely to the excluded volume of the protein (Tanford, 1961). The results obtained are given in Figure 2. For salts with large preferential hydrations the chemical potential of water, μ_1, after first following a decrease with protein concentration, increased sharply and finally became larger than that of pure water, μ_1^0. The protein concentration at which $\mu_1 - \mu_1^0 = 0$ should be close to C_{sat}, the solubility of the protein. Among the acetate salts, NaOAc was calculated to be the most effective in salting out BSA (C_{sat} = 0.14 g/mL), consistent with the general classification of this salt in the salting-out class. $Mg(OAc)_2$ and $Ca(OAc)_2$ also belong to the same class, their values of C_{sat} being ca. 0.28 and 0.32 g/mL, respectively. $Ba(OAc)_2$ should not salt out BSA at protein concentrations usually employed, its calculated value of C_{sat} being 1 g/mL. In the case of the SO_4^{2-} salts, the C_{sat} values for Na_2SO_4 and $MgSO_4$ (0.06 and 0.18 g/mL, respectively) are fully consistent with the classification of these salts in the strong salting-out category. Comparison of parts A and B of Figure 2 indicates that $MgSO_4$ may be as strong as NaOAc in its salting-out effectiveness. Although $NiSO_4$ and $MnSO_4$ gave values of C_{sat} of ca. 35 and 1.5 g/mL, respectively, they should not be considered as salting-out salts for BSA, just as $Ba(OAc)_2$. The phase isotherms for lysozyme, depicted in Figure 2C, resulted in similar values of C_{sat} for NaCl, $Mg(OAc)_2$, and $MgSO_4$, i.e., 0.6, 0.7, and 0.9 g/mL, respectively. The similar effectiveness of $MgSO_4$ and $Mg(OAc)_2$ is consistent with the results for BSA. The great

Table IV: Preferential Interaction Parameters of BSA in $MgCl_2$ at pH 4.5

salt concn (M)	ϕ_2^0 (mL/g)	$\phi_2'^0$ (mL/g)	$(\partial g_3/\partial g_2)_{T,\mu_1,\mu_3}$ (g/g)	$(\partial g_1/\partial g_2)_{T,\mu_1,\mu_3}$ (g/g)	$(\partial m_3/\partial m_2)_{T,\mu_1,\mu_3}$ (mol/mol)	$(\partial\mu_3/\partial m_3)_{T,P,m_2}$ [(cal/mol of salt)/ mol of salt]	$(\partial\mu_2/\partial m_3)_{T,P,m_2}$ [(cal/mol of protein)/ mol of salt]	ratio
0.5	0.733 ± 0.001	0.737 ± 0.001	−0.0049 ± 0.0025	0.102 ± 0.051	−3.51 ± 1.80	3760	13200 ± 6800	0.24
1	0.736 ± 0.001	0.739 ± 0.001	−0.0040 ± 0.0027	0.041 ± 0.027	−2.83 ± 1.89	2540	7200 ± 4900	0.13
1.5	0.737 ± 0.001	0.740 ± 0.001	−0.0042 ± 0.0028	0.029 ± 0.019	−3.02 ± 2.01	2240	6800 ± 4500	0.12
2	0.735 ± 0.001	0.737 ± 0.002	−0.0030 ± 0.0050	0.015 ± 0.022	−2.12 ± 3.18	2130	4500 ± 6800	0.08

Table V: Preferential Interaction Parameters of Proteins in $MgCl_2$ and $MgSO_4$

condition	app[a]	ϕ_2^0 (mL/g)	$\phi_2'^0$ (mL/g)	$(\partial g_3/\partial g_2)_{T,\mu_1,\mu_3}$ (g/g)	$(\partial g_1/\partial g_2)_{T,\mu_1,\mu_3}$ (g/g)	$(\partial\mu_2/\partial m_3)_{T,P,m_2}$ [(cal/mol of protein)/ mol of salt]	$(\partial\mu_2/\partial m_3)^{exptl}/(\partial\mu_2/\partial m_3)^{calcd}$	A_3[c] (g/g)
				β-LG ~ $MgCl_2$				
pH 2.0, 2 M[b]	P	0.750 ± 0.001	0.770	−0.0296 ± 0.0015	0.148 ± 0.007	23900 ± 1300	0.64	0.0304
pH 3.0, 1 M[b]	P	0.745 ± 0.001	0.761 ± 0.001	−0.0211 ± 0.0026	0.216 ± 0.027	21200 ± 2600	0.57	0.0082
2 M		0.750 ± 0.001	0.756 ± 0.001	−0.0089 ± 0.0030	0.044 ± 0.015	7200 ± 2400	0.19	0.0514
pH 5.1, 1 M		0.748 ± 0.001	0.750 ± 0.001	−0.0026 ± 0.0026	0.027 ± 0.027	2600 ± 2600	0.07	0.0267
2 M		0.751 ± 0.001	0.750 ± 0.001	0.0015 ± 0.0030	−0.007 ± 0.015	−1200 ± 2400	−0.03	0.0615
pH 7.0, 1 M		0.748 ± 0.001	0.749 ± 0.001	−0.0013 ± 0.0026	0.013 ± 0.026	1300 ± 2600	0.03	0.0280
2 M		0.750 ± 0.001	0.746 ± 0.001	0.0059 ± 0.0030	−0.030 ± 0.015	−4800 ± 2400	−0.13	0.0659
pH 7.9, 2 M		0.747 ± 0.002	0.745 ± 0.001	0.0030 ± 0.044	−0.015 ± 0.022	−2400 ± 3600	−0.06	0.0630
				β-LG ~ $MgSO_4$				
pH 3.0, 1 M[b]	P	0.744 ± 0.002	0.795 ± 0.001	−0.0670 ± 0.0039	0.554 ± 0.032	13700 ± 800	0.55	
pH 5.1, 1 M[b]	T	0.745 ± 0.001	0.785	−0.0524 ± 0.0013	0.433 ± 0.011	10700 ± 300	0.43	
				BSA ~ $MgCl_2$				
pH 3.0, 1 M	T	0.734 ± 0.001	0.746 ± 0.001	−0.0158 ± 0.0026	0.162 ± 0.027	29400 ± 4000	0.53	0.0135
2 M		0.739 ± 0.001	0.741 ± 0.001	−0.0029 ± 0.0029	0.015 ± 0.015	4300 ± 4300	0.08	0.0571
pH 8.8, 1 M		0.735 ± 0.001	0.733	0.0026 ± 0.0013	−0.027 ± 0.014	−4900 ± 2400	−0.09	0.0320
				BSA ~ $MgSO_4$				
pH 4.5, 1 M		0.734 ± 0.001	0.769 ± 0.001	−0.0469 ± 0.0027	0.388 ± 0.022	17700 ± 1000	0.48	
				Lysozyme ~ $MgCl_2$				
pH 3.0, 1 M[b]	P	0.708 ± 0.001	0.726 ± 0.001	−0.0238 ± 0.0020	0.244 ± 0.022	9300 ± 800	0.48	0.0054
pH 4.5, 1 M		0.712	0.721 ± 0.001	−0.0119 ± 0.0013	0.122 ± 0.013	4600 ± 500	0.24	0.0174
				Lysozyme ~ $MgSO_4$				
pH 3.0, 1 M[b]	P	0.708 ± 0.001	0.753 ± 0.001	−0.0591 ± 0.0026	0.488 ± 0.022	4700 ± 200	0.37	
pH 4.5, 1 M		0.709 ± 0.002	0.747 ± 0.001	−0.0498 ± 0.0039	0.412 ± 0.033	4000 ± 300	0.31	

[a] Appearance (app) of the protein solution was indicated as P and T, respectively, corresponding to "precipitation" and "turbidity". [b] Protein concentration was measured in 6 M Gdn·HCl. [c] $A_1 = 0.3$ g/g.

difference between $MgSO_4$ and $MgCl_2$ (Arakawa & Timasheff, 1982a) is manifested again in their values of C_{sat} (7 g/mL for $MgCl_2$ and 0.18 g/mL for $MgSO_4$when the protein is BSA).

Effects of $MgCl_2$ Concentration and Protein Charge. Since 1 M $MgCl_2$ showed essentially zero preferential interaction with BSA while $MgSO_4$ was strongly excluded from the protein domain, the preferential interactions of $MgCl_2$ were subjected to a more detailed examination. Namely, they were examined first as a function of salt concentration and second as a function of protein charge. Results, presented in Table IV, show that at pH 4.5 the preferential interaction parameter, $(\partial g_3/\partial g_2)_{T,\mu_1,\mu_3}$, remained close to zero up to 2 M $MgCl_2$, except for 0.5 M $MgCl_2$, where a small, but significant, preferential hydration was observed. A plot of $(\partial\mu_2/\partial m_3)_{T,P,m_2}$ vs. $MgCl_2$ concentration, shown in Figure 3A, suggests a gradual decrease of this parameter with salt concentration. When a protein hydration of 0.3 g/g was assumed, the total binding of $MgCl_2$ to BSA was calculated with eq 6. As shown in Figure 3B, this parameter increased linearly with $MgCl_2$ concentration, reaching 40 mol/mol at 2.0 M. Attempts at fitting this isotherm to the simple binding equation $A_3 = nka_3/(1 + ka_3)$, where n is the number of independent and identical binding sites, k is the binding constant, and a_3 is the activity of $MgCl_2$, failed for all values of n and k. A somewhat better fit was obtained when $a_\pm$ was used instead of a_3, suggesting that the $MgCl_2$ binding is occurring in ionic rather than in the ion pair

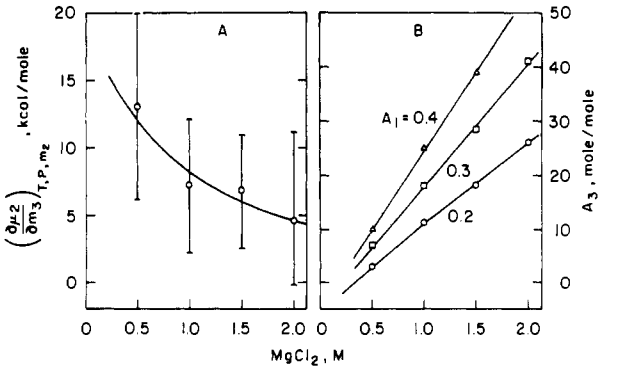

FIGURE 3: $MgCl_2$ concentration dependence of $(\partial\mu_2/\partial m_3)_{T,P,m_2}$ (A) and A_3 (B) for BSA in aqueous $MgCl_2$ system.

form. Nevertheless, the deviation was still significant, and no improvement was obtained by varying A_1 between 0.2 and 0.4 g/g. The failure to fit any of the A_3 values to the binding equation suggests either a dependence of the protein hydration on salt concentration or penetration of $MgCl_2$ at higher concentrations into the protein molecules due possibly to a partial loosening of the protein with the appearance of additional binding sites.

The effect of protein charge on preferential interactions was examined by varying the pH of the system. The results are listed in Table V. In some cases, as indicated in the table,

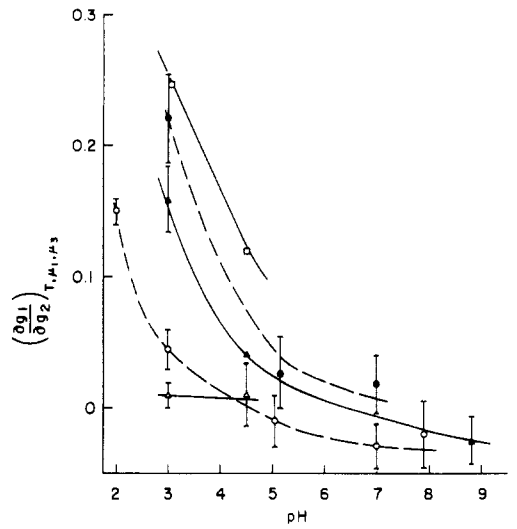

FIGURE 4: Dependence of $(\partial g_1/\partial g_2)_{T,\mu_1,\mu_3}$ on pH. Lysozyme (□), β-LG (●, ○), and BSA (▲, △) in $MgCl_2$. $MgCl_2$ concentration: 1 (□, ●, ▲) and 2 M (○, △).

the proteins were found to be not well soluble. The protein solutions developed more or less large aggregates in 1 M $MgCl_2$, pH 3.0, and for β-LG, also in 2 M $MgCl_2$, pH 2.0. In all cases, an increase in $MgCl_2$ concentration at the same pH or an increase in the solvent pH at the same $MgCl_2$ concentration led to full solubilization of the proteins. For all proteins tested, $(\partial g_3/\partial g_2)_{T,\mu_1,\mu_3}$ was negative at acid conditions and became close to zero within experimental error with an increase in pH. Figure 4 shows large preferential hydrations at acid conditions regardless of the protein used, in particular at pH 3.0 in 1 M $MgCl_2$. As the pH increases, this parameter decreases, tending toward zero between pH 4 and pH 6 for BSA and β-LG but remaining somewhat positive for lysozyme which carries a significant net positive charge in that pH range. Consequently, $(\partial\mu_2/\partial m_3)_{T,P,m_2}$ is large only at acid conditions; i.e., addition of $MgCl_2$ increases significantly the chemical potential of the proteins when these are positively charged. In fact, a comparison of the values of $(\partial\mu_2/\partial m_3)_{T,P,m_2}$ for $MgCl_2$ at pH 3.0 (29400 cal/mol for BSA and 9300 cal/mol for lysozyme; Table V) with those for NaCl (18300 cal/mol for BSA at pH 5.6 and 6800 cal/mol for lysozyme at pH 4.5; Table I) and for $MgSO_4$ at pH 4.5 (17700 cal/mol for BSA and 4000 cal/mol for lysozyme; Table V) shows higher values for $MgCl_2$. This relation is opposite to the relative values of the preferential hydration. The explanation of this apparent contradiction can be found in the exact relation between these two parameters, defined by eq 5 where $(\partial\mu_3/\partial m_3)_{T,P,m_2} = (nRT/m_3)[1 + (\partial \ln \gamma_\pm/\partial m_3)m_3]$. Thus, a difference in nonideality and n ($n = 2$ for $MgSO_4$ and $n = 3$ for $MgCl_2$: Robinson & Stokes, 1959) between two salts can lead to a drastic difference between their preferential bindings to a protein even though the chemical potential perturbations are identical. As shown in Table I, the values of $(\partial\mu_3/\partial m_3)_{T,P,m_2}$ are 4 times larger for $MgCl_2$ than for $MgSO_4$ at 1 M, with the consequence that a small value of the binding parameter $(\partial m_3/\partial m_2)_{T,\mu_1,\mu_3}$ leads to a larger value of $(\partial\mu_2/\partial m_3)_{T,P,m_2}$ for $MgCl_2$ than for $MgSO_4$. Conversely, equal perturbations of the free energy of a protein by $MgCl_2$ and $MgSO_4$ result in a smaller preferential interaction for $MgCl_2$ than for $MgSO_4$, and the variation of $(\partial\mu_2/\partial m_3)_{T,P,m_2}$ between different type of salts need not always be parallel to $(\partial m_3/\partial m_2)_{T,\mu_1,\mu_3}$. It is, therefore, the former parameter that should be used in comparing the thermodynamic effects of different salts, while the binding expression of the interaction is most useful in the direct

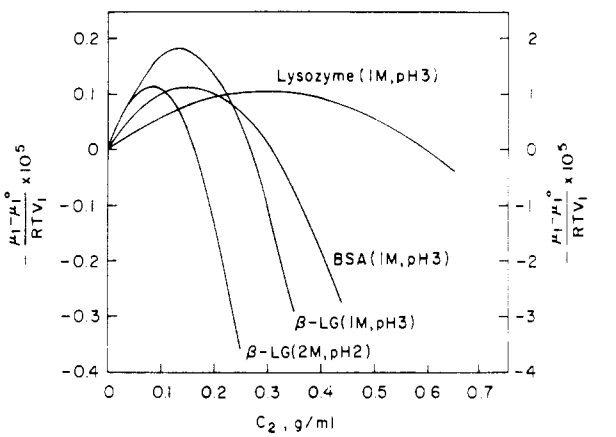

FIGURE 5: Phase isotherm for proteins in $MgCl_2$ at acidic pH. Left ordinate for β-LG and BSA. Right ordinate for lysozyme.

Table VI: Protein Solubility Estimated from Phase Isotherm

protein	C_{sat} (g/mL) pH 3.0	pH 4.5
	1 M $MgCl_2$	
BSA	0.31	
lysozyme	0.59	7.0
β-LG	0.27	
	2 M $MgCl_2$	
β-LG	0.16 (pH 2.0)	
	1 M $MgSO_4$	
BSA	HP[a]	0.18
lysozyme	0.60	0.94
β-LG	0.16	0.27 (pH 5.1)

[a] Heavy precipitation.

analysis of experimental observations measured on the concentration scale. One consequence of the large positive value of $(\partial\mu_2/\partial m_3)_{T,P,m_2}$ when the charge on the protein is positive is the sharp decrease in C_{sat} of proteins in $MgCl_2$ at acid pH, as shown by the phase isotherms of Figure 5 and the C_{sat} values summarized in Table VI. For $MgCl_2$ at or above pH 4.5, the phase isotherm indicated either no phase separation or a large value of C_{sat}. At acid conditions, the values of C_{sat} were reduced to 0.2–0.6 g/mL, i.e., to values comparable with those of the $MgSO_4$ and NaCl systems shown in Figure 2. This strong dependence of protein solubility on pH predicted from $(\partial\mu_2/\partial m_3)_{T,P,m_2}$ is fully consistent with the qualitative observation that in $MgCl_2$ at pH 4.5 the protein solutions were transparent, while at acid conditions all the protein solutions gave indications of precipitation.[6]

Contribution of Surface Free Energy Perturbation. The high preferential hydrations in the Na_2SO_4 and $MgSO_4$ systems, along with low or even negative values in other salts, indicate that the hydration observed in concentrated salt solutions is a complex phenomenon. It cannot be interpreted simply in terms of the sum of water molecules bound at such specific sites as charged groups, polar side chains, and peptide bonds. Possible sources of the variation in hydration could

[6] Since the protein concentrations used for the preferential interaction measurements were lower than C_{sat} calculated from the phase isotherm, no precipitation should have been expected. This discrepancy can be ascribed to the various assumptions introduced into the phase isotherm equation, such as the estimate of $(\partial\mu_2^{(e)}/\partial m_2)_{T,P,m_3}$ and the neglect of the higher terms. Furthermore, the particle weights of protein aggregates are higher than those used to calculate $(\partial m_3/\partial m_2)_{T,\mu_1,\mu_3}(\partial\mu_2/\partial m_3)_{T,P,m_2}$. This should affect that term much more than $(\partial\mu_2^{(e)}/\partial m_2)_{T,P,m_3}$ since M_2 appears in the second order in the former term and in the first order in the latter, leading to an overestimate of C_{sat}.

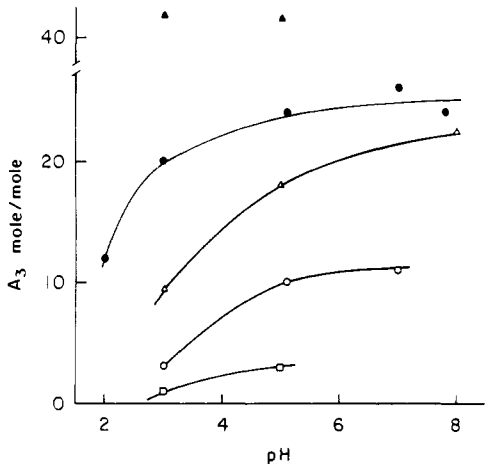

FIGURE 6: pH dependence of $MgCl_2$ binding to proteins: BSA in 1 (△) and 2 M (▲), β-LG in 1 (○) and 2 M (●), and lysozyme in 1 M (□).

be the effect of salts on the surface tension of water, i.e., on the free energy at the protein–water interface, and direct binding of the ions to specific sites on protein molecules.

The change in the surface free energy at protein–water interfaces induced by addition of the salts, $(\partial\mu_2/\partial m_3)^{\text{calcd}}_{T,P,m_2}$, was calculated from (Timasheff et al., 1976; Lee & Timasheff, 1981)

$$(\partial\mu_2/\partial m_3)^{\text{calcd}}_{T,P,m_2} = N_{\text{Av}} s_2 (\partial\sigma/\partial m_3)_{T,P,m_2} \quad (8)$$

where N_{Av} is Avogadro's number, s_2 is the surface area of a protein molecule, and σ is the surface tension of the aqueous salt solution. The ratio of the experimental value of $(\partial\mu_2/\partial m_3)_{T,P,m_2}$ to that calculated by eq 8, which is a measure of the contribution of the surface free energy perturbation to the preferential interactions, is listed in the last columns of Tables I and IV for pH values close to neutrality. The values of s_2 were calculated from the partial specific volumes of the proteins and their surface to volume ratios determined by small-angle X-ray scattering (Pessen et al., 1971; Luzzati et al., 1961). At pH values close to neutrality, only the Na^+ salts showed high values of the ratio, ca. 0.7. For $MgSO_4$, the ratio was lower, being 0.3– to 0.5, and for $MgCl_2$, it was even lower, in particular for BSA. For $CaCl_2$, this ratio was negative, despite the large surface tension increment characteristic of that salt. It is evident, therefore, that for the divalent cation salts the surface tension increment has no apparent direct relation to the observed preferential interactions, in contrast to the case of the monovalent cation salts (Arakawa & Timasheff, 1982a).

An understanding of the sources of this difference may be gleaned from a comparison of this ratio at acid pH and at neutrality. The results listed in the next to last column of Table V[7] reveal high values of this ratio, 0.5–0.6, for $MgCl_2$ at acid conditions, not much different from those for $MgSO_4$ at pH 4.5, although somewhat lower than those for the NaCl and Na_2SO_4 systems. This suggests an important role for the surface tension effect in the generation of preferential hydration. The pH dependence, however, indicates that other factors are also involved and that the mechanism of the preferential interactions of $MgCl_2$ and possibly other divalent

[7] The surface area of β-LG was calculated from its partial specific volume and the ratio of surface area to volume for BSA. Although β-LG is known to dissociate into subunit at pH 2.0, its dimer molecular weight of 36 800 was used throughout because of the presence of concentrated salt.

Table VII: Solubility of Amino Acids in Concentrated $MgCl_2$ and $MgSO_4$ Solutions[a]

	g of amino acid/100 g of solvent				
amino acid	water	1 M $MgCl_2$	2 M $MgCl_2$	1 M $MgSO_4$	2 M $MgSO_4$
glycine	22.7	27.8	32.5	26.0	25.7
L-leucine	2.14	2.24	2.14	1.57	0.568
L-tryptophan	1.23	1.38	1.36	0.898	0.428

[a] At 20 °C.

cation salts with proteins is more complicated than that proposed for such salts as NaCl and $NaSO_4$.

What is the source of the variation with pH of the preferential hydration in the $MgCl_2$ system? A calculation of the total binding of $MgCl_2$ with the assumption that total hydration is, in fact, water immobilized on the protein (A_1 = 0.3 g/g) gives a simple explanation of this phenomenon. The results in Figure 6 indicate that, for all cases, the value of A_3 decreases significantly with a decrease in pH, except for BSA in 2 M $MgCl_2$, suggesting that the observed preferential hydration at acid conditions is simply a reflection of the decrease in the total binding of $MgCl_2$ as the protein charge becomes more positive; in other words, it is a change in the balance of eq 6 in favor of hydration.

This was further probed by examining the difference in behavior between $MgCl_2$ and $MgSO_4$ as a function of pH. The results are given in Table V. In 1 M $MgSO_4$, BSA and lysozyme gave clear solutions at pH 4.5, β-LG was turbid at pH 5.1, and all three proteins showed some precipitation at pH 3.0. The heavy precipitation of BSA in fact made measurements impossible due to the instability of the density readings. At 2 M $MgSO_4$, pH 3.0, β-LG was even less soluble than in 1 M, in contrast to its behavior in $MgCl_2$ which, at the same pH, solubilized this protein completely when its concentration was increased to 2 M. Large protein preferential hydrations and, hence, positive values of $(\partial\mu_2/\partial m_3)_{T,P,m_2}$ were observed in $MgSO_4$ in all cases, $(\partial g_1/\partial g_2)_{T,\mu_1,\mu_3}$ being around 0.4–0.5 g/g. These parameters increased somewhat when the pH was lowered from 4.5 or 5.1 to 3.0, but to a much smaller extent than with $MgCl_2$. The ratio of $(\partial\mu_2/\partial m_3)^{\text{exptl}}_{T,P,m_2}$ to $(\partial\mu_2/\partial m_3)^{\text{calcd}}_{T,P,m_2}$ was higher for $MgSO_4$ than for $MgCl_2$ at pH 4.5 or 5.1, while at pH 3.0, the values were of similar magnitude in 1 M salt. The values of C_{sat} for this system, given in Table VI, indicate that the protein solubility in $MgSO_4$ may be decreased somewhat by lowering the pH, but to a much smaller extent than for $MgCl_2$.

Solubility. An explanation for the difference between the protein solubilization properties of $MgCl_2$ and $MgSO_4$ was sought in their effects on nonpolar amino acid side chains. Robinson & Jencks (1965a) have shown that the solubility of the model peptide ATGEE[1] in water is increased by salts such as KI, $BaCl_2$, and $CaCl_2$, whereas it is decreased by NaCl and Na_2SO_4, suggesting a difference in their affinities for the peptide unit. Since $MgCl_2$ had no effect on ATGEE solubility, it is evident that the salting-out effect of NaCl on ATGEE must be ascribed to the difference between Mg^{2+} and Na^+. One possibility is that the salting-out effects of both Mg^{2+} and Cl^- are zero, salting out being due to Na^+. Another is that both Na^+ and Cl^- have a salting-out effect. In that case, Mg^{2+} must have a salting-in effect which is balanced by the opposite effect of Cl^-. While Robinson & Jencks (1965a,b) did not carry out a comparison of $MgSO_4$ and $MgCl_2$, they have suggested that divalent cations may bind to peptide bonds in the same manner as Gdn·HCl and urea, while Nozaki & Tanford (1963, 1970) have shown that the last two compounds act also through their affinity to hydrophobic side chains. The

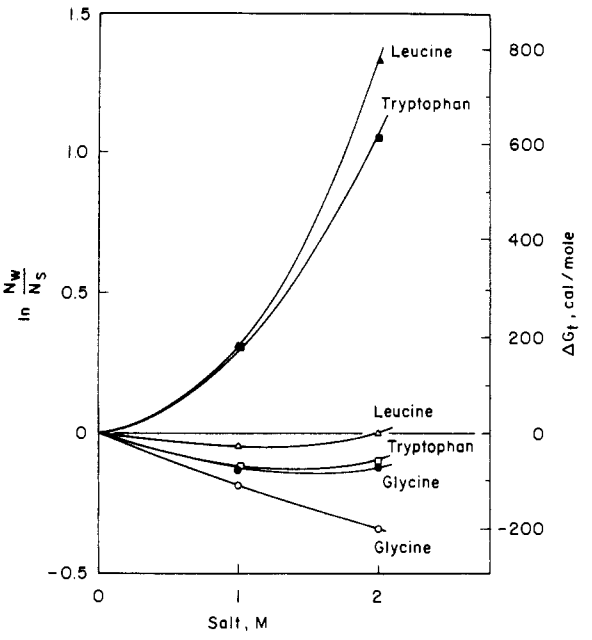

FIGURE 7: Concentration dependence of amino acid solubility (N_s) and transfer free energy (ΔG_t) in $MgCl_2$ (open symbol) and $MgSO_4$ (full symbol).

suggestion that the effect of salts on these side chains may depend on the ionic species has prompted us to measure the solubility of nonpolar amino acids in $MgCl_2$ and $MgSO_4$. The results are presented in Table VII.

Two amino acids were chosen as representative of hydrophobic side chains, L-leucine and L-tryptophan. The solubilities were found to be high in both salts relative to those in organic cosolvents. The solubility of glycine was greater in both $MgCl_2$ and $MgSO_4$ than in water. That of leucine was essentially unchanged by the addition of $MgCl_2$, while it was decreased greatly by $MgSO_4$. In the case of L-tryptophan, the solubility increased somewhat in $MgCl_2$, but it decreased greatly in $MgSO_4$. From these measurements, the transfer free energies of the amino acids from water to the salt solution, ΔG_t, were calculated from (Nozaki & Tanford, 1963)

$$\Delta G_t = \mu^0_s - \mu^0_w = RT \ln N_w/N_s + RT \ln \gamma_w/\gamma_s \quad (9)$$

where s and w refer to the salt solution and water, respectively, N is the solubility of the amino acid expressed in mole fraction, and γ is the activity coefficient. Since γ is a function principally of the amino acid concentration (Nozaki & Tanford, 1963), and the solubilities of the amino acids in the salt solutions are not very much different from those in water, $\gamma_w \simeq \gamma_s$, and the second term on the right hand side of eq 9 may be neglected. The dependence of ΔG_t on salt concentration is shown in Figure 7. In all cases, there is an upward curvature. Following the assumptions of Nozaki & Tanford (1963, 1965, 1970, 1971) that the transfer free energies of the peptide group and the side chain are additive and that the transfer free energy for the glycine side chain is zero, the transfer free energy of any given side chain may be obtained from

$$\Delta G_t^{side} = \Delta G_t^{amino\ acid} - \Delta G_t^{glycine} \quad (10)$$

As is evident from Figure 7, the transfer free energies of the leucine and tryptophan side chains from water to the salt solutions are positive; i.e., the interactions of these side chains with the salt solutions at high salt concentrations are more unfavorable than those with water. The difference in ΔG_t both of leucine and of tryptophan from that of glycine is much larger for $MgSO_4$ than for $MgCl_2$. For example, in 2 M salt, the transfer free energies of the leucine and tryptophan side chains are respectively 200 and 140 cal/mol for $MgCl_2$ and 840 and 680 cal/mol for $MgSO_4$. The large difference between the two salts must be ascribed to their anionic species.

Discussion

From the present and previous studies (Arakawa & Timasheff, 1982a), it is evident that preferential interactions are a thermodynamic measure of the effects of salts on the stability and solubility of proteins. Such salts as $BaCl_2$, $CaCl_2$, and $MgCl_2$, known as protein destabilizers and salting-in salts (von Hippel & Schleich, 1969), showed little preferential hydration, just as did KSCN (Arakawa & Timasheff, 1982a, and unpublished results), while $MgSO_4$, known as a salting-out salt (Cohn & Edsall, 1943), showed a large preferential hydration, as did Na_2SO_4. $MnCl_2$ and $NiCl_2$ can be added to the salting-in class, probably as even stronger destabilizers, while $Mg(OAc)_2$ and $Ca(OAc)_2$ should be listed as salting-out salts and $MnSO_4$ and $NiSO_4$ cannot be classified.

The measured preferential interactions cover a wide spectrum, ranging from large preferential hydration to preferential salt binding, depending not only on the kind of salt but also on the solvent conditions. The first question to be asked is what is the cause of the large differences between the salts in their preferential interactions with proteins? Preferential interaction results alone, being thermodynamic measurements, cannot lead to mechanistic conclusions. This question can be approached, however, by making some simple assumptions. One approach has been to assume that the protein hydration in the presence of the cosolvent is equal to that determined directly by various techniques, ~0.3 g/g, and to calculate the total binding of the additive to the protein by eq 6. The binding mechanism can then be defined by correlating the total cosolvent binding calculated with the number of expected binding sites, as has been done successfully for Gdn·HCl (Lee & Timasheff, 1974) and for urea (Prakash et al., 1981). For the salt systems, however, this method has led to negative values of total salt binding in some cases such as Na_2SO_4, NaOAc, and $MgSO_4$, meaning that there must, in fact, be exclusion of the salt from the protein. Such negative values of total cosolvent binding have been calculated also for some sugar and amino acid systems (Arakawa & Timasheff, 1982b, 1983).

Hydration values higher than normal hydration have found an explanation in terms of the surface tension effect (Lee & Timasheff, 1981; Timasheff et al., 1976; Arakawa & Timasheff, 1982a,b). Since all the salts have positive surface tension increments in water, it can be expected that all will be excluded from proteins (see eq 8). Although the NaCl and Na_2SO_4 systems showed a reasonable agreement with this prediction, the experimental value of the salt exclusion was always somewhat lower than the calculated one. Such a deviation is not surprising in view of the use of surface tension increments measured at flat water–air interfaces to calculate the exclusion at a curved protein–water interface. It may also be an indication of salt binding to the protein (Scatchard & Black, 1949; Scatchard et al., 1950, 1957, 1959; Klotz, 1952; Steinhardt & Reynolds, 1969), as inferred for the KSCN, $MgCl_2$, and $CaCl_2$ systems from their total disagreement with calculation from the surface tension effect (Arakawa & Timasheff, 1982a). It is clear, therefore, that the observed preferential interactions of salts with proteins should be considered in terms of a summation of the various phenomena that can take place at the protein–solvent interface, namely, in terms of overall salt bindings and exclusions. In this model, the hydration value, A_1, must be calculated from the surface tension increment of the salt.

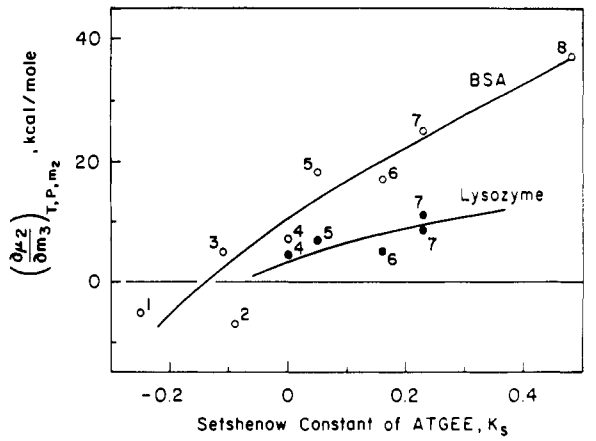

FIGURE 8: Correlation between preferential interaction parameter and the salting-out constant on ATGEE: $(\partial\mu_2/\partial m_3)_{T,P,m_2}$ from BSA and lysozyme in 1 M salt and 1.4 M glycine. 1, KSCN (Arakawa & Timasheff, 1982a); 2, $CaCl_2$; 3, $BaCl_2$; 4, $MgCl_2$; 5, NaCl; 6, glycine (Arakawa & Timasheff, 1983); 7, CH_3COONa (for lysozyme; Arakawa & Timasheff, 1982a); 8, Na_2SO_4.

Robinson & Jencks (1965a) have determined the salting-out constants of various salts for ATGEE. This effectiveness increases as a whole with an increase of the surface tension increment of the salt but with deviations for divalent cation salts, just as observed for the protein preferential interactions. Figure 8 shows plots of $(\partial\mu_2/\partial m_3)^{\text{exptl}}_{T,P,m_2}$ for BSA and lysozyme vs. the salting-out constant for ATGEE, indicating a distinct correlation between the proteins and the model compound. In this plot, the protein results at pH 4.5 were used, since at this pH the proteins have low net charges, which approximate better ATGEE which has no charged groups. The correlation of Figure 8 suggests that similar mechanisms are involved in the salt interactions with proteins and ATGEE. Schrier & Schrier (1967) have attempted to eliminate the effect of salt binding to peptide bonds from the overall effect of salts on the solubility of model compounds and have obtained salting-out constants for CH_2 groups. This parameter varies from one salt to another in a manner consistent with their surface tension increments but with a systematic deviation that depends on the cationic species. This result is consistent with our proposal, with the modification that the interaction of salts with CH_2 groups may also make a small contribution to deviations from the surface tension effect.

The present results have shown that the transfer free energy of nonpolar side chains is unfavorable in both $MgCl_2$ and $MgSO_4$ solutions, a situation similar to the solubility of acetone (Long & McDevit, 1952). This could be expected from the positive surface tension increments of these salts. Since this increment is higher for $MgCl_2$ than for $MgSO_4$, the larger unfavorable transfer free energy for the latter salt suggests a favorable salt interaction with these side chains, which is considerably greater for $MgCl_2$ than for $MgSO_4$. The surface free energy changes for these side chains, induced by the addition of the salts, were calculated by using $(\partial\sigma/\partial m_3)_{T,P,m_2}$ = 3.16 and 2.10 for $MgCl_2$ and $MgSO_4$, respectively, and the surface areas of the side chains, which were approximated from the difference in the partial molar volumes of leucine and tryptophan from that of glycine and by taking a spherical approximation for these residues. Assuming that $(\partial\mu_s/\partial m_3)^{\text{calcd}}_{T,P,m_3}$ (where s designates side chains) is independent of m_3, the transfer free energies of the side chains from water to 2 M salt, μ_s(2 M salt) − μ_s(water), were calculated to be, in the case of $MgCl_2$, 1300 and 900 cal/mol for tryptophan and leucine, respectively (the values obtained from solubility were 140 and 240 cal/mol), and, in the case of $MgSO_4$, 900 and 600 cal/mol, respectively (the experimental values were 680 and 840 cal/mol). For $MgSO_4$, the calculated values were comparable with the experimental ones. The great difference in the case of $MgCl_2$ suggests a large contribution of favorable interactions for $MgCl_2$. One possibility is that $Mg^{2+}\cdot XH_2O$ interacts favorably with these side chains as does Gdn·H^+, with little perturbation by Cl^- for $MgCl_2$ but with large, almost total, perturbation by SO_4^{2-}. These results support the concept that the protein preferential interactions with salts arise from two opposing factors, namely, the surface tension effect contributing to an unfavorable free energy change and the salt binding to peptide bonds and some side chains as major sources of favorable free energy. The differences in effects between the Na^+ and divalent cation salts and between $MgSO_4$ and $MgCl_2$ at pH 4.5 or 5.1 would be due then to the differences in their binding to proteins.

Quantitatively this can be expressed by decomposing the experimental value of the preferential interaction into the exclusion due to the surface tension effect, $(\partial m_3/\partial m_2)^{\text{excl}}_{T,\mu_1,\mu_3}$, and the actual salt binding, $(\partial m_3/\partial m_2)^{\text{bind}}_{T,\mu_1,\mu_3}$:

$$(\partial m_3/\partial m_2)^{\text{obsd}} = (\partial m_3/\partial m_2)^{\text{excl}}_{T,\mu_1,\mu_3} + (\partial m_3/\partial m_2)^{\text{bind}}_{T,\mu_1,\mu_3} \quad (11)$$

where the signs of the two quantities on the right-hand side are always opposite. By use of this relation, the actual binding of the salts was calculated from the difference between the experimental preferential interaction and the exclusion expected from eq 8 and 5. For BSA, for example, the surface tension increment alone predicts that 1 M $MgCl_2$ and $MgSO_4$ should be excluded from the protein domain to the extents of 23 and 55 mol/mol, respectively. The observed values of $(\partial m_3/\partial m_2)_{T,\mu_1,\mu_3}$ were −3 and −27 mol/mol, giving by difference binding values of 20 and 28 mol/mol for $MgCl_2$ and $MgSO_4$, respectively. The larger calculated value of the binding for $MgSO_4$ than for $MgCl_2$ indicates that the overall contribution of SO_4^{2-} to the salt binding is greater than that of Cl^-, if the contribution of Mg^{2+} is identical in $MgSO_4$ and $MgCl_2$. This result is not specific to BSA, since the same calculations give binding values of 6 and 13 mol/mol of $MgCl_2$ and $MgSO_4$, respectively, to lysozyme and of 14 and 21 mol/mol to β-LG. It is evident, therefore, that although the binding of $MgSO_4$ to proteins is greater than that of $MgCl_2$, it is its value relative to the exclusion due to the surface tension effect (for BSA, 28 mol vs. 55 mol) that leads to the significant preferential hydration in the $MgSO_4$ system. In the case of $MgCl_2$ the binding is of similar magnitude to the exclusion (20 and 23 mol, respectively), resulting in the nearly total cancellation of the two factors and an insignificant preferential hydration. This is what renders these two salts greatly different in their preferential interactions with proteins. The same calculation for the NaCl and Na_2SO_4 systems gave bindings of 10 mol/mol to BSA for both, i.e., considerably lower than for the Mg^{2+} salts, explaining the better correlation of the Na^+ salts with the surface tension effect.

The change in the net charge on the protein at lower pH can also alter the overall salt bindings. The binding of 1 M $MgCl_2$ to BSA at pH 3.0, calculated as described above, was 10 mol/mol (compared to 20 mol/mol at pH 4.5 or 5.1). Similar calculations gave values of 4 mol/mol $MgCl_2$ and 12 mol/mol $MgSO_4$ bound to lysozyme at pH 3.0, compared to 6 and 13 mol/mol at pH 4.5. For β-LG the calculation gave 7 and 17 mol/mol bound for $MgCl_2$ and $MgSO_4$, respectively, at pH 3.0, compared to 14 and 21 mol/mol at pH 4.5. The lower values of salt binding at pH 3 indicate that the decrease in the binding of Mg^{2+} due to the increased net positive charge of the protein was larger than the increase in the binding of the anionic species. In other words, the large binding of the

Mg^{2+} salts observed at neutral pH stemmed mainly from the contribution of Mg^{2+}. The larger decrease in binding for $MgCl_2$ than for $MgSO_4$ when the pH was lowered suggests that SO_4^{2-} binding increases more than Cl^- binding when the net positive charge on the protein increases. The small decreases in the binding of both salts to lysozyme are consistent with its high isoelectric point, i.e., 11 (Alderton et al., 1945) vs. ca. 5 for β-LG (Treece et al., 1964) and BSA (Foster, 1960), and hence a smaller increase in the net positive charge with a decrease in pH. It is evident, therefore, that the better correlation at low pH between the observed preferential interactions and the surface tension effect is due to the decreases in the binding of the Mg^{2+} salts. Consistent with this analysis is the observation that, at all pHs studied, an increase in $MgCl_2$ concentration to 2 M resulted in an increase of the preferential binding, most evident at pH 3.0, with disappearance of the preferential hydration already at 1 M and an increase in total binding of salt (Figure 6). At 2 M $MgCl_2$, lowering the pH to 2 restored the preferential hydration as the net positive charges of the proteins increased further.

One of the important findings of this study is that the preferential hydration parameter increases in the order of $Cl^- < OAc^- < SO_4^{2-}$ for the divalent cation salts, just as for the Na^+ salts, in full agreement with the Hofmeister series of anions. The perturbation of the activity of the proteins, $(\partial\mu_2/\partial m_3)_{T,P,m_2}$, also follows essentially this order, with the exception that in some cases $(\partial\mu_2/\partial m_3)_{T,P,m_2}$ of the SO_4^{2-} salts is lower than that of the OAc^- salts, as is seen in Table I. The difference between the Na^+ and the divalent cation salts arises simply from the difference in their values of $(\partial\mu_3/\partial m_3)_{T,P,m_2}$, i.e., from differences in the nonideality of the salts. This quantity varies little in the Na^+ salts, but it increases in the order of $SO_4^{2-} \ll OAc^- < Cl^-$ for the divalent cation salts. As a result, the preferential interaction parameter is nearly parallel to $(\partial\mu_2/\partial m_3)_{T,P,m_2}$ for the Na^+ salts but not for the divalent cation salts. In fact, $(\partial m_3/\partial m_2)_{T,\mu_1,\mu_3}$ for BSA in the Mg^{2+} salts decreased in the order of $Cl^- < OAc^- < SO_4^{2-}$ whereas $(\partial\mu_2/\partial m_3)_{T,P,m_2}$ increased in the order of $Cl^- < SO_4^{2-} < OAc^-$. The phase isotherms, calculated from the $(\partial\mu_2/\partial m_3)_{T,P,m_2}$ values, predicted that the salting-out effectiveness increases in the order of $MgCl_2 \ll Ca(OAc)_2 < Mg(OAc)_2 < MgSO_4$ for BSA and $MgCl_2 < MgSO_4 \leq Mg(OAc)_2$ for lysozyme; i.e., protein solubility should follow closely the Hofmeister series of anions even for the divalent cation salts, with possible small deviations.

A further correlation between protein solubility and the preferential interaction parameter is afforded by the physical appearance of the proteins in the salt solutions. For example, the appearance of turbidity, as in 1 M $MgCl_2$, pH 3.0, was accompanied by a large preferential hydration, while solubilization in 2 M $MgCl_2$, pH 3.0, was accompanied by a sharp drop in preferential hydration. It seems very pertinent that conditions exist at which $MgCl_2$ can precipitate all the proteins examined. Nevertheless, that salt cannot be classified as a strong salting-out salt since it is a general rule for such salts that an increase in salt concentration decreases sharply the protein solubility. This is contrary to observation when the $MgCl_2$ concentration is increased from 1 to 2 M. On the other hand, when the $MgSO_4$ concentration was increased from 1 to 2 M at pH 3.0, β-LG showed an enhanced precipitation. Furthermore, for true salting-out salts such as NaCl and $MgSO_4$, preferential interactions are little dependent on pH, while for $MgCl_2$, this dependence is strong.

The effect of preferential interaction on denaturation and association reactions of macromolecules can be understood best in terms of the Wyman linkage relation (Wyman, 1964):

$$(\partial \ln K/\partial \ln a_3)_{T,P,m_2} = (\partial m_3/\partial m_2)^{P}_{T,\mu_1,\mu_3} - (\partial m_3/\partial m_2)^{R}_{T,\mu_1,\mu_3} = \Delta\nu_3 - (m_3/55.5)\Delta\nu_1 = \Delta\nu \quad (12)$$

where R and P refer to the reactant and product, respectively, K is the equilibrium constant of the reaction, and $\Delta\nu_3$ and $\Delta\nu_1$ are the differences between the two states in the bindings of additive and water, respectively, in moles per mole of protein. Since denaturation in general results in an increase both of the number of binding sites and of the surface area, $\Delta\nu_3$ and $\Delta\nu_1$ should be positive, and $\Delta\nu$ is determined by a balance of the two values, as well as by m_3. For salts such as Na_2SO_4, NaCl, and $MgSO_4$, which show large protein preferential hydrations, the increase on denaturation of the protein surface area and hence in the value of ν_1 as a reflection of increased salt exclusion will be more significant than $\Delta\nu_3$, leading to negative values of $\Delta\nu$; i.e., addition of these salts will decrease the value of K and stabilize the native form. On the other hand, for those salts that show little or negative preferential hydrations, contribution of $\Delta\nu_3$ may be larger than that of $\Delta\nu_1$, leading to positive values of $\Delta\nu$ and enhancement of the denaturation. These include all the divalent cation salts of Cl^-. For association reactions, R and P are the monomeric protein and the protein in the aggregate, respectively, and K is the association constant. Since the protein–protein contacts decrease the number of binding sites, as well as the surface area per monomer, both $\Delta\nu_3$ and $\Delta\nu_1$ should be negative. When the same arguments as used for denaturation are applied, for salts which show large preferential hydrations the second term will be more negative than the first, leading to positive values of $\Delta\nu$; i.e., addition of these salts should enhance the self-association. In association reactions, however, specific effects such as the loss of highly hydrated sites with a more negative $\Delta\nu_1$ than expected must be added to the general considerations, the effect of salts reflecting then both their general effectiveness and the types of specific forces involved in the intersubunit contacts.

Registry No. NaCl, 7647-14-5; NaOAc, 127-09-3; Na_2SO_4, 7757-82-6; $MgCl_2$, 7786-30-3; $Mg(OAc)_2$, 142-72-3; $MgSO_4$, 7487-88-9; $CaCl_2$, 10043-52-4; $Ca(OAc)_2$, 62-54-4; $BaCl_2$, 10361-37-2; $Ba(OAc)_2$, 543-80-6; $MnCl_2$, 7773-01-5; $MnSO_4$, 7785-87-7; $NiCl_2$, 7718-54-9; $NiSO_4$, 7786-81-4; lysozyme, 9001-63-2; glycine, 56-40-6; L-leucine, 61-90-5; L-tryptophan, 73-22-3.

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