ABSTRACT: The preferential interactions of proteins with solvent components were studied in concentrated salt by densimetric measurements. Proteins were found to be preferentially hydrated in NaCl, NaCH₃CO0, and Na₂S0₄. The resulting unfavorable free-energy change was related to the effects of these salts on solubility and stability of the proteins. This unfavorable free-energy change was correlated with the large, positive surface tension increment of these salts, i.e., their perturbation of surface free energy. On the other hand, KSCN, CaCl₂, and MgCl₂ showed considerable binding to bovine serum albumin, which could be related to their destabilizing and salting-in effects on macromolecules. Since the last two salts have high surface tension increments, it was concluded that this does not necessarily lead to protein preferential hydration and stabilization.

The conformational stability and solubility of proteins are sensitive functions of solvent composition. The effect of salts on these properties of proteins in aqueous solution is a strong function of the ionic species present. For example, CaCl₂ and NaSCN are known to decrease the conformational stability of macromolecules (von Hippel & Wong, 1965) and to have a salting-in effect (von Hippel & Schleich, 1969; Klotz, 1965), while high concentrations of (NH₄)₂S0₄ and Na₂S0₄ are commonly used to precipitate or crystallize proteins in the native form. This topic has been reviewed in detail by von Hippel & Schleich (1969).

Previous studies from our laboratory have shown that knowledge of preferential interactions of solvent components with proteins in three-component systems can lead to an understanding of the mechanism of the effect of solvent components on the stability and solubility of proteins (Lee & Timasheff, 1974, 1981; Timasheff & Inoue, 1968; Lee et al., 1975; Timasheff et al., 1976; Gekko & Timasheff, 1981; Pittz & Timasheff, 1978). It seemed of interest, therefore, to investigate whether the various salt effects on proteins can also be related to the preferential interaction patterns of salts with proteins. Kuntz & Kauzmann (1974) have reviewed the available data on the effect of salt on protein hydration, but the information is insufficient to interpret the widely different salt effects in terms of preferential interactions.

In their analysis of the effect of solvents on the stability of DNA, Sinanoglu & Abdulnur (1964, 1965) have proposed an important role for the free energy of cavity formation reflected in the surface tension of the solvent. This effect has been found (Lee & Timasheff, 1981; Arakawa & Timasheff, 1982) to make a significant contribution to protein stabilization by sugars in aqueous solution, the preferential interaction being a strong function of the increase in the surface tension of water by the addition of sucrose (International Critical Tables, 1928). Recently, Melander & Horvath (1977) have concluded on the basis of the cavity theory that the solubility of proteins in aqueous salt solutions can be corrected with the surface tension increment of water induced by the addition of salts. We have undertaken, therefore, a study of the preferential interactions of proteins with solvent components in aqueous salt solutions in order to explore possible relationships between such interactions and the stability and solubility of proteins as well as with the surface tension effects, and the results are presented in this paper.

Materials and Methods

Bovine serum albumin (BSA)¹ (lot 65C-7533) and lysozyme (lot 57C 8025) were purchased from Sigma. All the salts used, CH₃COONa, NaCl, Na₂S0₄, KSCN, MgCl₂, CaCl₂, and MgSO₄, were of reagent grade and were used without further purification.

The preferential interactions of the solvent components with proteins were obtained from the partial specific volumes of the proteins, measured with a Precision DMA-02 density meter (Anton Paar, Graz), using previously described procedures (Lee & Timasheff, 1974; Lee et al., 1979; Gekko & Timasheff, 1981) and the protocol of the preceding paper (Arakawa & Timasheff, 1982). The preferential interaction parameter, \( \frac{\Delta g_i}{\Delta g_2} \) is obtained from the data by (Casassa & Eisenberg, 1964)

\[
\frac{\Delta g_i}{\Delta g_2} = \frac{1 - \rho_i \phi_i^2}{1 - \rho_2 \phi_2^2}
\]

where \( g_i \) is the concentration of component \( i \) in grams of \( i \) per gram of water, \( \mu_i \) is its chemical potential, \( T \) is the Kelvin temperature, \( \rho_0 \) is the density of the reference solvent, \( \phi_i \) is the partial specific volume of component 3 (the salt), and \( \phi_i^2 \) and \( \phi_2^2 \) are the apparent partial specific volumes of the protein determined at conditions at which the osmotic and the chemical potential of component 3 are, in turn, kept identical in the solution and the reference solvent. The preferential interaction parameter is a direct expression of changes in the free energy of the system induced by addition of component 3, since

\[
\frac{\Delta g_i}{\Delta g_2} = \frac{\partial \mu_i}{\partial m_i} \bigg|_{T,P,m_2} - \frac{\partial \mu_3}{\partial m_2} \bigg|_{T,P,m_2} \frac{\partial m_2}{\partial m_3} \bigg|_{T,P,m_2} \frac{\partial m_3}{\partial m_3} \bigg|_{T,P,m_2} (2)
\]

where \( P \) is pressure and \( m_i \) is the molar concentration of component \( i \) related to \( g_i \) by \( m_i = 1000g_i/M_i \) where \( M_i \) is its molecular weight. The preferential solvation by component 3 is related to preferential hydration by

\[
\frac{\Delta g_1}{\Delta g_2} = -\frac{\Delta g_3}{\Delta g_2} \bigg|_{T,P,m_3} \frac{\partial g_3}{\partial g_2} \bigg|_{T,P,m_3} (3)
\]

1 Abbreviation: BSA, bovine serum albumin.

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The identity, within experimental error, of the \( \phi_2 \) values in various solutions with the value in dilute buffer strongly supports the assumption on the identity of the absorptivity values. This is further supported by the measured values for lysozyme cited above.

\[^a\] Dunn (1966).

The cosolvent self-interaction term, \( \left( \frac{\partial \mu_3}{\partial m_1} \right)_{T,P,m_3} \), can be calculated from

\[
\left( \frac{\partial \mu_3}{\partial m_1} \right)_{T,P,m_3} = RT \left( \frac{\partial \ln a_3}{\partial m_1} \right)_{T,P,m_3} \tag{3}
\]

\( a_3 \) can, in turn, be calculated from the mean molal activity coefficient, \( \gamma_3 \), of salt with eq 4 for NaCl, CH\(_3\)COONa, KSCN, and MgSO\(_4\) and with eq 5 for CaCl\(_2\), Na\(_2\)SO\(_4\), and MgCl\(_2\). The partial specific volumes of the proteins were measured in a 0.02 M acetate or phosphate buffer, except for the CH\(_3\)COONa and CaCl\(_2\) systems. The acetate solutions were equimolar in CH\(_3\)COONa and CH\(_3\)COOH. For example, the 0.5 M acetate solution contained 0.5 M CH\(_3\)COONa and 0.5 M CH\(_3\)COOH. The pH of a 1 M CaCl\(_2\) solution was adjusted with HCl.

Protein concentrations were determined on a Cary Model 118 spectrophotometer. The absorbance values used were 27.4 1. \( \mathrm{dL}/(\mathrm{g} \, \mathrm{cm}) \) at 281 nm for lysozyme (Roxby & Tanford, 1971) and 6.58 1. \( \mathrm{dL}/(\mathrm{g} \, \mathrm{cm}) \) at 278 nm for BSA (Noelken & Timasheff, 1967) in dilute salt solutions. The absorbance values of the proteins in concentrated solutions are expected to be somewhat different from those of the native proteins in dilute salt. For lysozyme, these were determined by diluting volumetrically two aliquots of aqueous protein solution with identical amounts of a dilute buffer in which the absorptivity of the protein is known and of salt solution in question and then measuring their absorbances at 281 nm. The resulting absorbance values were 27.2 1. \( \mathrm{dL}/(\mathrm{g} \, \mathrm{cm}) \) in 0.5 and 1 M acetate and 27.7 1. \( \mathrm{dL}/(\mathrm{g} \, \mathrm{cm}) \) in 1 M NaCl. For BSA, the dilute salt absorbance value was used throughout. While this may affect slightly the absolute values of the partial specific volumes, it should have no effect on the preferential interaction parameters.\(^2\) The light scattering contribution was found to be insignificant and was neglected. Where needed, the partial

\[ a_3 = m_3 \gamma_3^2 \tag{4} \]

\[ a_3 = 4m_3 \gamma_3^3 \tag{5} \]

Results

The partial specific volumes of BSA and lysozyme were measured in dilute buffer, both at constant molality and at constant chemical potential. The results, listed in Table I, show that the two values were close to identical in both cases, indicating that the experimental procedures were satisfactory for an examination of the preferential interactions of the salts with proteins, since the interaction parameter is obtained from the difference between the partial specific volumes at the two conditions in the presence of the salt. Typical plots of the apparent specific volume, \( \phi_{app} \) vs. protein concentration are shown in Figure 1. While for lysozyme, \( \phi_2 \) increased slightly with protein concentration at all conditions, BSA showed little concentration dependence. In no case was there a concentration dependence of \( \phi_2 \).

![Figure 1](https://example.com/figure1.png)

**FIGURE 1:** Protein concentration dependence of the apparent specific volume: lysozyme, 1 M acetate (O, ■), 0.5 M acetate (right-side solid square); BSA, 1 M NaCl, pH 4.5 (O, ●), 1 M Na\(_2\)SO\(_4\), pH 4.5 (△, △).

### Table I: Interaction Parameters

<table>
<thead>
<tr>
<th>Condition</th>
<th>( \psi_3 ) (g/g)</th>
<th>( \psi_2 ) (g/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.02 M acetate, pH 4.5</td>
<td>0.714 ± 0.002</td>
<td>0.712 ± 0.001</td>
</tr>
<tr>
<td>0.5 M acetate, pH 4.68</td>
<td>0.0431 ± 0.050</td>
<td>0.712 ± 0.002</td>
</tr>
<tr>
<td>1 M acetate, pH 4.71</td>
<td>0.0906 ± 0.016</td>
<td>0.712 ± 0.002</td>
</tr>
<tr>
<td>1 M NaCl, pH 4.5</td>
<td>0.0597 ± 0.033</td>
<td>0.707 ± 0.002</td>
</tr>
<tr>
<td>1 M Na(_2)SO(_4), pH 4.5</td>
<td>0.735 ± 0.001</td>
<td>0.736 ± 0.001</td>
</tr>
<tr>
<td>0.5 M Na(_2)SO(_4), pH 4.5</td>
<td>0.0718 ± 0.165</td>
<td>0.734 ± 0.001</td>
</tr>
<tr>
<td>0.5 M Na(_2)SO(_4), pH 7.0</td>
<td>0.145 ± 0.208</td>
<td>0.735 ± 0.002</td>
</tr>
<tr>
<td>1 M Na(_2)SO(_4), pH 5.6</td>
<td>0.141 ± 0.208</td>
<td>0.735 ± 0.002</td>
</tr>
<tr>
<td>1 M NaCl, pH 4.5</td>
<td>0.0596 ± 0.331</td>
<td>0.734 ± 0.001</td>
</tr>
<tr>
<td>1 M NaCl, pH 5.6</td>
<td>0.0597 ± 0.331</td>
<td>0.735 ± 0.001</td>
</tr>
<tr>
<td>1 M CaCl(_2), pH 5.6</td>
<td>0.114 ± 0.216</td>
<td>0.734 ± 0.001</td>
</tr>
<tr>
<td>1 M KSCN, pH 5.6</td>
<td>0.102 ± 0.216</td>
<td>0.735 ± 0.001</td>
</tr>
<tr>
<td>0.5 M MgCl(_2), pH 4.5</td>
<td>0.0479 ± 0.150</td>
<td>0.733 ± 0.001</td>
</tr>
<tr>
<td>1 M MgCl(_2), pH 5.6</td>
<td>0.0975 ± 0.176</td>
<td>0.737 ± 0.001</td>
</tr>
<tr>
<td>1 M Na(_2)SO(_4), pH 4.5</td>
<td>0.121 ± 0.136</td>
<td>0.734 ± 0.001</td>
</tr>
</tbody>
</table>

\[ \frac{\partial \mu_3}{\partial m_1} = RT \left( \frac{\partial \ln a_3}{\partial m_1} \right)_{T,P,m_3} \]
the contribution of the preferential interactions of CH₃COOH with the proteins to the difference between φ'/₀ and φ'/₂, should be much smaller than that of CH₃COONa, the partial specific volume of CH₃COOH (0.864 mL/g) being much closer to that of water than that of CH₃COONa (0.516 mL/g) at 1 M. Assuming 0.04 g/g for the preferential binding of CH₃COOH in 1 M acetate, which would correspond to a very strong interaction, we obtain 0.708 mL/g for φ'/₂ which is not much different from the experimental value of 0.712 mL/g for φ'/₀. It may be reasonably concluded, therefore, that the observed difference is mostly due to interaction of CH₃COONa with the protein. The value of (dg/dg')ₜ,ₚ,ₚ' was negative in all cases for CH₃COONa, NaCl, and Na₂SO₄, indicating a deficiency of the salt in the immediate domain of the protein, namely, a preferential hydration of the protein, as shown in the last column of Table I. In the case of MgCl₂, the measured preferential hydration was very small and essentially within experimental error from zero. To the contrary, MgSO₄, known as a strong salting-out agent, showed a large preferential hydration, the magnitude of which was between those of NaCl and Na₂SO₄. For KSCN and CaCl₂, (dg/dg')ₜ,ₚ,ₚ' was small and positive, but within experimental error from zero, indicating that there is probably a small excess of these two salts in the domain of the protein.

The preferential interaction parameter is related to the total values of the binding of water and salt to the proteins by (Inoue & Timasheff, 1972)

$$\left( \frac{dg}{dg'} \right)_{T,\mu,\mu'} = A_3 - g_3 A_1$$

where A₁ and A₂ are the total amounts of water and salt bound to protein, respectively, expressed as grams of each component per gram of protein. For CH₃COONa, NaCl, and Na₂SO₄, assuming total exclusion of the salt from the domain of the protein, namely, setting A₂ = 0, (dg/dg')ₜ,ₚ,ₚ' is equal to the total hydration, A₁. All the values given in the last column of Table I are close to, or greater than, the usual protein hydration value of 0.2-0.4 g of water per g of protein (Kuntz & Kauzmann, 1974; Kuntz, 1971; Bull & Breese, 1968), indicating that these salts may be largely excluded from the domain of the protein. For KSCN, MgCl₂, and CaCl₂, the amount of salt binding to the protein, A₂, was calculated with the assumption of a hydration of 0.3 g/g of protein (A₁ = 0.3). The results are shown in the seventh column of Table I and in the third column of Table II. They indicate a significant salt binding to proteins.

The effect of the salts on the chemical potentials of the proteins, (dμ₁/dm₁)ₜ,p,mₙ₁, was calculated with eq 2 and is given in the fifth column of Table II. The cosolvent self-interaction term, (dμ₃/dm₃)ₜ,p,mₙ₃, listed in the sixth column, was calculated with eq 3-5, by using values of τₚ taken from Robinson & Stokes (1955). The large positive change in chemical potential seen for CH₃COONa, NaCl, and Na₂SO₄ shows that introduction of the salt into an aqueous solution of the proteins is thermodynamically unfavorable, destabilizing the system. This may lead to association or aggregation of the protein, or even to phase separation in the form of amorphous precipitation or crystallization. Quantitatively, the last effect may be expressed in terms of a phase isotherm, which is the dependence of the chemical potential of the principal solvent on protein concentration (Pittz & Timasheff, 1978; Timasheff et al., 1976):
<table>
<thead>
<tr>
<th>Condition</th>
<th>( \frac{\partial m_s}{\partial m_z} ) at ( T, \mu, \mu_z )</th>
<th>( A_z ) (mol/mol)</th>
<th>( \frac{\partial a}{\partial m_z} ) at ( T, P, m_z )</th>
<th>( \frac{\partial \mu_s}{\partial m_z} ) exp</th>
<th>( \frac{\partial \mu_s}{\partial m_z} ) cal</th>
<th>( \frac{\partial \mu_s}{\partial m_z} ) exp</th>
<th>( \frac{\partial \mu_s}{\partial m_z} ) cal</th>
<th>( C_{\text{satn}} ) (g/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5 M acetate, pH 4.68</td>
<td>-5.14 ± 1.10</td>
<td>11380 ± 2440</td>
<td>2214</td>
<td>0.30</td>
<td>0.25</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 M acetate, pH 4.71</td>
<td>-7.53 ± 1.19</td>
<td>8500 ± 1340</td>
<td>1126</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 M NaCl, pH 4.5</td>
<td>-6.20 ± 1.54</td>
<td>1.64</td>
<td>6750 ± 1680</td>
<td>0.678</td>
<td>0.45</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Lysozyme**

<table>
<thead>
<tr>
<th>Condition</th>
<th>( \frac{\partial m_s}{\partial m_z} ) exp</th>
<th>( \frac{\partial m_s}{\partial m_z} ) cal</th>
<th>( \frac{\partial m_s}{\partial m_z} ) exp</th>
<th>( \frac{\partial m_s}{\partial m_z} ) cal</th>
<th>( C_{\text{satn}} ) (g/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5 M Na₂SO₄, pH 4.5</td>
<td>2.73</td>
<td>21480 ± 2700</td>
<td>2179</td>
<td>47500</td>
<td>0.452</td>
</tr>
<tr>
<td>0.5 M Na₂SO₄, pH 7.0</td>
<td>14.2 ± 1.86</td>
<td>30940 ± 4050</td>
<td>2179</td>
<td>47500</td>
<td>0.651</td>
</tr>
<tr>
<td>1 M Na₂SO₄, pH 4.5</td>
<td>-32.0 ± 2.1</td>
<td>33630 ± 2210</td>
<td>1051</td>
<td>47500</td>
<td>0.708</td>
</tr>
<tr>
<td>1 M Na₂SO₄, pH 5.6</td>
<td>-35.4 ± 1.3</td>
<td>37210 ± 1370</td>
<td>1051</td>
<td>47500</td>
<td>0.783</td>
</tr>
<tr>
<td>1 M NaCl, pH 4.5</td>
<td>-18.4 ± 3.7</td>
<td>18280 ± 4030</td>
<td>1088</td>
<td>28700</td>
<td>0.698</td>
</tr>
<tr>
<td>1 M NaCl, pH 5.6</td>
<td>-16.8 ± 3.7</td>
<td>18280 ± 4030</td>
<td>1088</td>
<td>28700</td>
<td>0.637</td>
</tr>
<tr>
<td>1 M CaCl₂, pH 5.6</td>
<td>2.25 ± 1.50</td>
<td>23.3</td>
<td>3.66</td>
<td>-5160 ± 3440</td>
<td>2292</td>
</tr>
<tr>
<td>1 M KSCN, pH 5.6</td>
<td>4.94 ± 3.29</td>
<td>26.4</td>
<td>0.45</td>
<td>-4910 ± 3270</td>
<td>994</td>
</tr>
<tr>
<td>0.5 M MgCl₂, pH 4.5</td>
<td>-3.51 ± 1.80</td>
<td>6.8</td>
<td>3.16</td>
<td>13210 ± 6780</td>
<td>3764</td>
</tr>
<tr>
<td>1 M MgCl₂, pH 5.6</td>
<td>-2.97 ± 1.98</td>
<td>17.9</td>
<td>2.16</td>
<td>7650 ± 5100</td>
<td>2577</td>
</tr>
<tr>
<td>1 M MgSO₄, pH 4.5</td>
<td>-26.5 ± 1.5</td>
<td>2.10</td>
<td>200.30 ± 1130</td>
<td>756</td>
<td>36500</td>
</tr>
</tbody>
</table>

\( a \) In units of dynes per centimeter per mole of salt in 1000 g of H₂O. \( b \) In units calories per mole of salt per moles of \( i \) in 1000 g of H₂O.
where \( S \) is the protein solubility in grams per milliliter, \( C_i \) is the salt concentration in moles per liter, \( K_s \) is the salting-out constant, and \( \beta \) is the solubility extrapolated to zero salt concentration, but which does not correspond to the actual solubility, but a higher value. It is of interest to note that at 1 M NaCl and NaSO\(_4\), this ratio does not vary much from a value of 0.7, independent of the nature of both the protein and the salt. For MgCl\(_2\), the experimental value, 7650 cal/mol, is only 14% of the calculated one, and, for KSCN and CaCl\(_2\), the calculated and experimental values are of opposite signs. In view of the constancy of this ratio over a variety of conditions, deviations from this ratio may be taken as a measure of the solvent stabilization–ion binding balance. A strong downward deviation may be regarded as the overcoming of preferential exclusion by specific binding of the ionic species.

The effects of pH and nature of the salts on the interaction parameter for BSA seem particularly striking. BSA is known to undergo a partial unfolding in acid pH (Yang & Foster, 1954; Aoki & Foster, 1957; Vigai & Foster, 1967). Comparison of the values of the ratio in column 8 of Table II shows that at pH 4.5 and 0.5 M NaSO\(_4\) this ratio is lowered to 0.452, suggesting penetration of the salt into the domain of the protein and possible salt binding. This effect is not observed in 0.5 M NaSO\(_4\) at pH 7.0, in 1 M NaSO\(_4\) at pH 4.5, or in 1 M NaCl at pH 4.5, suggesting that it is the anion which is mainly responsible for the thermodynamic stabilization and that it is required at a high molar level. In the case of the magnesium salts, the preferential exclusion is perturbed by the binding of the Mg\(^{2+}\) ion to BSA (Robinson & Jencks, 1965). As a result, the ratio is lowered. Comparing 0.5 M MgCl\(_2\) with 1 M NaCl, both at pH 4.5 and 1 M in Cl\(^-\), shows that the presence of Mg\(^{2+}\) ions greatly reduces the ratio. The same situation is true of the pair of 1 M MgSO\(_4\)–1 M NaSO\(_4\), where the levels of the stabilizing sulfate ion are identical. Yet in the magnesium salt, the SO\(_4^{2-}\) ion is not able to counteract fully the direct binding effect of Mg\(^{2+}\) to BSA. The MgSO\(_4\)–MgCl\(_2\) pair shows that the SO\(_4^{2-}\) ion is a greater structure stabilizer than Cl\(^-\), since in this case the ratio is greater for MgSO\(_4\), even though the measurement was done at lower pH. This is consistent with the comparison of NaSO\(_4\) with NaCl. Both at identical anion concentrations (1 M) and at identical Na\(^+\) concentrations (1 M), and at pHs where the protein is quite stable, the exclusion of the salt from the protein is stronger for SO\(_4^{2-}\), as is evident from column 5 of Table II.
Discussion

The results presented in this paper show that the preferential interaction with proteins of NaCl, CH₃COONa, and Na₂SO₄ is largely negative; i.e., the proteins are preferentially hydrated. Protein preferential hydration in concentrated salts has also been found for ribonuclease in 1.33 M Na₂SO₄ (Timasheff et al., 1976) and for chicken heart glyceroldehyde-3-phosphate dehydrogenase in potassium phosphate (Aune & Timasheff, 1970), which is known to be a strong salting-out salt. The universality of this observation leads to the conclusion that protein preferential hydration is a general phenomenon in salting-out systems. The introduction of these salts into an aqueous solution of the protein results in a positive change in the chemical potential of the protein, which should lead to an eventual phase separation. The phase isotherms and the values of $C_{stat}$ in Table II show that, at the protein concentrations commonly used, lysozyme and BSA cannot be precipitated by high concentrations of these salts except for 1 M Na₂SO₄. For example, lysozyme would precipitate at a concentration of more than 0.45 g/mL in 1 M NaCl, as shown in Figure 2. This is in agreement with the known fact that both proteins are highly soluble in aqueous media. Comparison of Figures 2 and 3 shows that lysozyme is more soluble than BSA. For both proteins, an increase in salt concentration predicts a lowering of the solubility, consistent with the salting-out precipitation of proteins. The salt concentration dependence of protein solubility appears to be stronger for Na₂SO₄ than for CH₃COONa, which might suggest that, in the 1 M acetate system, the interaction of CH₃COOH with the protein can no longer be neglected. As shown in Figures 2 and 3, and Table II, the protein solubility increases in the orders CH₃COONa < NaCl and Na₂SO₄ < NaCl < glucose when compared at the same molarity of the cosolvent. Figure 3 shows that, in 1 M cosolvent, BSA should precipitate at concentrations above 60 mg/mL for Na₂SO₄, 280 mg/mL for NaCl, and 430 mg/mL for glucose. The values of $C_{stat}$ indicate that 1 M Na₂SO₄ and 1 M acetate are, respectively, 4 times and twice as effective on protein solubility as 1 M NaCl, giving a salting-out order of effectiveness of NaCl < CH₃COONa < Na₂SO₄. These results are fully consistent with the known facts that Na₂SO₄ is a much stronger protein precipitant than NaCl and that CH₃COONa is intermediate between the two. There are no solubility data for proteins in aqueous sugar solutions. We may use, however, the data of Klotz (1965) on the effects of various substances on the clouding point of poly(vinylxazolidinone) in aqueous solution. In this system, sucrose was intermediate between (NH₄)₂SO₄ and NaCl. Considering the higher effectiveness of Na₂SO₄ as a precipitant than of (NH₄)₂SO₄, it seems reasonable to assume that mono- and disaccharides, such as glucose and sucrose, respectively, should be close to NaCl in their effectiveness as protein precipitants.

It has been reported by many authors (von Hippel & Schleich, 1969; Klotz, 1965) that the addition of KSCN, MgCl₂, or CaCl₂ to an aqueous solution of macromolecules results in the destabilization of the macromolecules, for example, in a decrease of their transition temperature. For these salts, the preferential interaction parameter, $(\partial g_1/\partial g_2)_{T,P,m}$, was found to be very small, in contrast to that for NaCl, CH₃COONa, and Na₂SO₄, permitting extensive total binding of these salts when calculated with the assumption of a hydration of 0.3 g of water per g of protein. Scatchard and co-workers have measured the binding of various salts to human serum albumin by different methods (Scatchard & Black, 1949; Scatchard et al., 1950, 1957, 1959). From measurements of the pH displacement of isionic protein solutions induced by the addition of salts, they found that as many as 25 mol of SCN⁻ was bound per mol of protein at 0.2 M salt. Our calculated value of 26 mol of salt/mol of protein for the total binding of KSCN to BSA is, therefore, quite consistent with these results, although we have used higher concentrations of the salt. Edsall et al. (1950) have also reported the binding of several Ca²⁺ ions to BSA at a low CaCl₂ concentration and considerable more Ca²⁺ binding sites than 10 at high concentration. Since in the present paper, the CaCl₂ interaction was measured at high salt concentration, the obtained result of 23 mol of salt/mol of protein again seems to agree with the direct measurement. Under the conditions used, it is highly unlikely that these salts bind to the proteins in the form of ion pairs; rather, it must be the cation or the anion which binds at specific sites, with the counterions distributed properly in the electrical double layer to satisfy the electroneutrality of the protein component. In the case of MgCl₂ and CaCl₂, the ions bound predominantly to the protein should be Mg²⁺ and Ca²⁺, since Cl⁻ is also a constituent of NaCl, which is mostly excluded from the domain of the protein, although chloride is known to bind to proteins (Scatchard et al., 1957). In the case of KSCN, both K⁺ and SCN⁻ are known to bind to proteins, but it is likely that it is the SCN⁻ which binds predominantly at specific sites on BSA. For these anions, the binding constants for human serum albumin (Scatchard et al., 1957) were reported to be 2400 (mol/1000 g of H₂O)⁻¹ for Cl⁻ and 46 200 (mol/1000 g of H₂O)⁻¹ for SCN⁻ on the first class of binding sites and to decrease for the second and third classes, but always remaining greater for SCN⁻ than for Cl⁻. There seem to be no reliable data for cation binding constants for both bovine and human serum albumins. It might be possible then to assign the destabilizing effects of KSCN, MgCl₂, and CaCl₂ to the binding of their constituent ion species to proteins. The increased net charge of the protein due to the binding of the ion should increase the electrostatic free energy of the protein, the resulting repulsive forces causing a decrease in the stability of the protein (Linderstrom-Lang, 1924). This electrostatic repulsive force should also prevent protein association or aggregation; in other words, it should increase the solubility of the proteins in aqueous solutions of these salts, which is fully in agreement with the known protein salting-in property of these salts.

Since surface tension has been invoked as an important factor contributing to protein solubility (Melander & Horvath, 1977), it seemed of interest to examine whether it could make a significant contribution to the preferential interaction of salts with proteins, as it does in the sucrose and glucose systems (Lee & Timasheff, 1981; Arakawa & Timasheff, 1982). In the NaCl and Na₂SO₄ solvent systems, the value of $(\partial g_1/\partial m_2)_{T,P,m}$ calculated from the surface tension increment induced by the salt, was found to be of the same order of magnitude as $(\partial g_1/\partial m_2)_{T,P,m}$ obtained from interaction data. It would seem, therefore, that, in these salts, the increase of the surface tension of water by addition of the salt can make a significant contribution to preferential interaction, just as in the case of sucrose and glucose (Lee & Timasheff, 1981; Arakawa & Timasheff, 1982). The surface area of a protein, however, may not necessarily contribute uniformly to the interface free energy. While $(\partial g_1/\partial m_2)_{T,P,m}$ for 1 M NaCl and 1 M Na₂SO₄ does not depend on the solvent pH, that for 0.5 M Na₂SO₄ at pH 4.5 is lower than those for the same salt concentration at pH 7.0 or for 1 M Na₂SO₄ at both pHs, indicating the contribution of other factors to $(\partial g_1/\partial m_2)_{T,P,m}$, e.g., the charge density on the surface of the protein.
In the case of CaCl₂, MgCl₂, and KSCN, there was no relationship between \( \left( \frac{\partial \mu_j}{\partial m_j} \right)_{T, P, m} \) and \( \left( \frac{\partial \mu_j}{\partial m_j} \right)_{T, P, m} \) of these quantities being of opposite signs for KSCN and CaCl₂. For the KSCN system, this result is reasonable, because, for this salt, the parameter \( \left( \frac{\partial \sigma}{\partial m_j} \right)_{T, P, m} \) is much lower than that for the other salts. The results with the MgCl₂ and CaCl₂ systems clearly point to the danger of assigning preferential hydration solely to the perturbation of the surface free energy by the salt, since both MgCl₂ and CaCl₂ have large values of \( \left( \frac{\partial \sigma}{\partial m_j} \right)_{T, P, m} \) and evidently other factors must be predominant.

It was J. Traube (Traube, 1910) who first pointed out the relation between the surface tension of solvents and the solubility of gases, nonelectrolytes, colloids, and suspensions in aqueous salt solution. He arranged anions and cations into separate series in order of their effectiveness in increasing the surface tension of the solvent and found a close relation between this effectiveness and the salt effect on the solubility of such substances. Although he did not give any theoretical interpretation for this result, his finding for anions is quite reliable because the relative effectiveness of anions on solubility, assigned by him, agrees with current knowledge. Recently, Melander & Horvath (1977) applied the cavity formation theory to aqueous salt solutions and proposed that protein solubility in aqueous salt solutions can be explained in terms of the surface tension of the solvent, as there is a good correlation between the molal surface tension increments of salts and the anionic lyotropic series. From the above two studies and our experiments, it seems evident that, in the salts with identical cations (in this study, CH₃COONa, NaCl, Na₂SO₄, and probably KSCN because of the similarity of K⁺ to Na⁺), there is a distinct relation between surface tension, solubility, and preferential interactions. This relation, however, does not hold fully, since CaCl₂ and MgCl₂ are classified as having a salting-in or destabilizing effect on proteins in spite of their higher surface tension increments than Na₂SO₄. Our finding that CaCl₂ is preferentially bound to proteins while MgCl₂ is only weakly excluded is fully consistent with their known salting-in properties. Since the increase in the surface tension of the solvent induced by the salts should lead to the exclusion of the salt components from contact with the protein, there must be some attractive forces between the salt and the protein which overcome the repulsive force due to the surface tension increment.

In this respect, the result for MgSO₄ is very suggestive. This salt, contrary to MgCl₂, showed a large preferential hydration, consistent with its high surface tension increment. Its value of the \( \left( \frac{\partial \mu_j}{\partial m_j} \right)_{T, P, m} \) \( \left( \frac{\partial \mu_j}{\partial m_j} \right)_{T, P, m} \) ratio, however, was significantly lower than those for NaCl and Na₂SO₄ indicating an important role of Mg²⁺ in the decrease of this ratio for MgCl₂ and MgSO₄. Thus, our results provide further evidence that the preferential interaction parameter is a good measure of the effectiveness of a solvent component as a protein structure stabilizer or destabilizer, as well as a salting-in or salting-out agent.

References
Isolation of Neuronal Parvalbumin by High-Performance Liquid Chromatography. Characterization and Comparison with Muscle Parvalbumin†

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ABSTRACT: Neuronal parvalbumin has been isolated from rat brain and purified to homogeneity by high-performance liquid chromatography (HPLC) on reverse-phase supports. This procedure includes four consecutive chromatographic steps with an overall protein recovery of 74% and a 26 400-fold purification. The concentration of parvalbumin was found to be approximately 10 mg/kg wet weight in brain tissue, which is about 100 times lower than that in rat muscle. The physical properties of brain parvalbumin are described and compared with those of the muscle counterpart. These proteins were identical in their molecular weights (12 000), isoelectric points (4.9), retention times on C-18 reverse-phase HPLC columns, Ca²⁺ content (two per molecule), amino acid compositions, and immunological properties. A comparison of the tryptic peptide maps of brain and muscle parvalbumin by analytical HPLC also revealed identity and showed that the isolation method described here did not alter the chemical structure of the protein.

The role of calcium in cellular regulation is thought to be mediated by Ca²⁺ receptors such as calmodulin, troponin C, S-100 protein, vitamin D dependent Ca²⁺-binding protein (CaBP),¹ and parvalbumin. Their Ca²⁺-binding sites display significant internal structural homology, a fact that has suggested evolution from a smaller ancestral precursor by gene duplication [for reviews, see Kretsinger (1980) and Siegel et al. (1980)]. Whereas calmodulin is involved in regulating a broad spectrum of cellular activities and troponin in regulating muscle contraction, the physiological role(s) of S-100, vitamin D dependent CaBP, and parvalbumin are less clear. The physical properties of muscle parvalbumins have been mainly described for lower vertebrates and more recently for mammals [for reviews, see Pechère et al. (1973), Hamoir (1974), and Kretsinger (1980)]. When antibodies against rat muscle parvalbumin are used, immunological cross-reactivity has only been detected in a few organs, including the brain from lower vertebrates (Gosselin-Rey et al., 1978; Gerday et al., 1979), mammals (Baron et al., 1975), and chicken (Heizmann & Strehler, 1979). Using a monospecific antiserum against rat muscle parvalbumin, Celio & Heizmann (1981) found that the immunologically active material in rat brains is restricted to a distinct subpopulation of neurons.

Characterization of the immunological active components present in brain preparations has not been attempted probably because of the small quantities present, e.g., estimated to be as low as 2 mg/kg of rabbit brain (Baron et al., 1975). Due to the usual poor overall yields experienced with the more "conventional" isolation methods (ion exchange, gel filtration, etc.), quite large amounts of starting material would be required for the preparation of milligram quantities of such proteins. Based on our previous observations concerning the chromatographic behavior of peptides (Hughes et al., 1979; Wilson et al., 1981a) and proteins (Wilson et al., 1982a, b) on reverse-phase supports, we felt that such methodology might well offer the possibility to prepare enough of the material to carry out a comparative study with muscle parvalbumin. The isolation of rat brain parvalbumin by high-performance liquid chromatography (HPLC) is described, and the amino acid analysis, two-dimensional gel pattern, and Ca²⁺ content as well as the immunological properties are compared with those of the muscle counterpart. In addition, HPLC, a sensitive and efficient tool to carry out peptide analysis in the subnanomole range (Wilson et al., 1981b), was chosen to prove the similarity (if not identity) of both proteins.

Materials and Methods

Proteins and Chemicals. Parvalbumin from leg muscles of 60-day-old rats (SIV-50) was prepared as described for the chicken protein (Strehler et al., 1977). The single alteration in the procedure was the addition of the following protease inhibitors to the homogenization medium: pepstatin (1 μM), PMSF (0.4 mM), TPCK (0.15 mM), leupeptin (1 μM), all

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Abbreviations: HPLC, high-performance liquid chromatography; CaBP, vitamin D dependent Ca²⁺-binding protein; PMSF, phenylmethanesulfonyl fluoride; TPCK, L(-)-tosylamide-2-phenylethyl chloromethyl ketone; trypsin C, Ca²⁺-binding subunit of troponin; EDTA, ethylenediaminetetraacetate; EGTA, ethylene glycol bis(β-aminoethyl ether)-N,N,N',N'-tetraacetate; Tris, tris(hydroxymethyl)aminomethane.