Hamilton, W. C. (1965) *Acta Crystallogr. 18,* 502.
Hinckley, C. C. (1969) *J. Am. Chem. Soc. 91,* 5160.
Ikeda, K., & Hamaguchi, K. (1973) *J. Biochem. (Tokyo) 73,* 307.
Imoto, I., Johnson, L. N., North, A. C. T., Phillips, D. C., & Rupley, J. A. (1972), *Enzymes, 3rd Ed., 7,* 665.
James, T. L. (1975) *Nuclear Magnetic Resonance in Biochemistry,* Academic Press, New York, N.Y.
Jolles, P., Juarigini-Adell, J., & Jolles, J. (1964) *C. R. Acad. Sci. 258,* 3926.
Jori, G., Gennari, G., Galliazzo, G., & Scaffone, E. (1971) *Biochim. Biophys. Acta 236,* 749.
Kurachi, K., Sieker, L. C., & Jensen, L. H. (1975) *J. Biol. Chem. 250,* 7663.
La Mar, G. N., Horrocks, W. De W., Jr., & Holm, R. H., Ed. (1973) *NMR of Paramagnetic Molecules: Principles and Applications,* Academic Press, New York, N.Y.
Marinetti, T. D. (1975) Ph.D. Dissertation, Harvard University, Cambridge, Mass.
Marinetti, T. D., Snyder, G. H., & Sykes, B. D. (1975) *J. Am. Chem. Soc. 97,* 6562.
Marinetti, T. D., Snyder, G. H., & Sykes, B. D. (1976) *Biochemistry 15,* 4600.
Marinetti, T. D., Snyder, G. H., & Sykes, B. D. (1977) *Biochemistry 16,* 647.
Marquardt, D. W. (1963) *J. Soc. Indust. Appl. Math. 11,* 431.
McConnell, H. M., & Robertson, R. E. (1958) *J. Chem. Phys. 29,* 1361.
McDonald, C. C., & Phillips, W. D. (1969) *Biochem. Biophys. Res. Commun. 35,* 43.
O'Neill, R. (1971) *J. R. Stat. Soc., Ser. C (Appl. Stat.) 20,* 338, Algorithm AS47.
Ostroy, F., Gams, R. A., Glickson, J. D., & Lenkinski, R. E. (1978), *Biochim. Biophys. Acta* (submitted for publication).
Phillips, D. C. (1967) *Proc. Natl. Acad. Sci. U.S.A. 57,* 757.
Reuben, J. (1973) *Prog. Nucl. Magn. Reson. Spectrosc. 9,* 1.
Reuben, J. (1976) *J. Am. Chem. Soc. 98,* 3726.
Rupley, J. A. (1967) *Proc. R. Soc. London, Ser. B 167,* 416.
Sievers, R. E., Ed. (1973), *Nuclear Magnetic Resonance Shift Reagents,* Academic Press, New York, N.Y.
Smith, L., Mohr, L., & Raftery, M. (1973) *J. Am. Chem. Soc. 95,* 7497.
Sophianopoulos, A. J., Rhodes, C. K., Holcomb, D. N., & Van Holde, K. E. (1962) *J. Biol. Chem. 237,* 1107.
Warshel, A., & Levitt, M. (1976) *J. Mol. Biol. 103,* 227.
Webb, G. A. (1970) *Annu. Rep. Nucl. Magn. Reson. Spectrosc. 3,* 211.
Willcott, M. R. W., III, & Davis, R. E. (1975) *Science 190,* 850.
Willcott, M. R. W., III, Lenkinski, R. E., & Davis, R. E. (1972) *J. Am. Chem. Soc. 94,* 1742.
Wüthrich, K. (1976) *NMR in Biological Research: Peptides and Proteins,* American Elsevier, New York, N.Y.
Yonath, A., Smilansky, A., & Sharon, N. (1974) *FEBS Lett. 49,* 178.

Effects of Hofmeister Salts on the Self-Association of Glucagon†

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ABSTRACT: The trimerization constants of glucagon at pH 10.6 in 0.76 M K_2HPO_4 have been calculated from circular dichroism data between 5 and 50 °C. The free energy, enthalpy, and entropy of transfer have been evaluated from the current results and published data in 0.20 M phosphate. The free energies of transfer from 0.20 to 0.76 M phosphate are negative at all temperatures investigated. The negative free energies of transfer are derived completely from an increase in the entropy of transfer, since the enthalpy of transfer is less favorable at all temperatures. These parameters are compared with those of various model groups and compounds: CH_2, peptide, methane, ethane, and the 1–13 N-terminal fragments of ribonuclease. The effects of fluoride and chloride on the self-association of glucagon have been compared with that of phosphate at 25 °C. These effects are consistent with the binding of approximately one molecule of salt to the trimer and a systematic decrease in the number of water molecules bound to the trimer compared to the monomer for the series K_2HPO_4, KF, and KCl.

In dilute solutions glucagon is largely unstructured, with few intramolecular contacts (Blanchard and King, 1966). At moderate concentrations a largely α-helical trimer is formed in alkali (Gratzer and Beaven, 1969). Cross-linking experiments with dimethyl suberimidate have demonstrated that the associated species is a trimer; no dimers or hexamers were found (Gratzer et al., 1972). It is also known that glucagon is a trimer in its crystalline state (Sasaki et al., 1975). Previous measurements of the self-association behavior of glucagon are consistent with a monomer $\rightleftharpoons$ trimer equilibrium (Formisano et al., 1977).

Since the self-association in alkali can be conveniently measured by several methods and the structure of the trimer is known from x-ray analysis (Sasaki et al., 1975), this association can serve as a useful model to evaluate the influence of the solvent composition on the equilibria involved in protein interactions. Since the x-ray data reveal that the trimer is

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stabilized by hydrophobic interactions between two nonpolar clusters of side chains at positions 6–14 and 22–29, solutes (or solvents) which affect these interactions should shift the association equilibrium.

The Hofmeister series of anions reduce the solubility of nonpolar gases and molecules containing nonpolar moieties (von Hippel and Schleich, 1969). Schrier and Schrier (1967), Nandi and Robinson (1972a,b), and von Hippel and Hamabata (1973) have shown that it is the nonpolar groups of small peptides which are responsible for their salting-out by Hofmeister anions, since the solubility of the peptide groups is increased. The Hofmeister anions should therefore modify the self-association of glucagon, since the reaction is driven by hydrophobic interactions (Blanchard and King, 1966; Formisano et al., 1977).

For most proteins, the Hofmeister anions fall into the following order: $PO_4^{2-} > SO_4^{2-} > F^- > Cl^- > Br^- > ClO_4^- > CNS^-$ (von Hippel and Schleich, 1969). A study of the temperature dependence of glucagon self-association in 0.20 M K_2HPO_4 has recently been published (Formisano et al., 1977). We now present results on the effect of K_2HPO_4 (0.76 M) on the thermodynamic parameters of this equilibrium. We have also compared the effects of fluoride and chloride with phosphate at room temperature. The salting-in anions such as CNS^- and ClO_4^- could not be evaluated readily due to the very weak association of glucagon *in water*.

Materials and Methods

Crystalline glucagon was obtained from Elanco Products Co. (a division of Eli Lilly and Co.) and from Sigma. Solutions were prepared and the CD measurements were made as described recently (Formisano et al., 1977).

Apparent molar association constants were evaluated by assuming that the total concentration of monomer could be expressed by a simple monomer $\rightleftharpoons$ trimer equilibrium, eq 1:

$$[C_t] = [C_m] + 3K_a[C_m]^3 \quad (1)$$

where $[C_t]$ is the total concentration expressed in monomer units, $[C_m]$ is the free monomer concentration, and K_a is the molar trimerization constant.

The free energy of association was expressed as a series expansion in temperature, eq 2:

$$\Delta G^\circ = A + BT + CT^2 + DT^3 \quad (2)$$

and

$$K_a = e^{-(A+BT+CT^2+DT^3)/RT} \quad (3)$$

The observed mean residue ellipticity $[\theta]$ is related to that of the monomer (E_m) and trimer (E_t) by

$$[\theta] = \frac{[C_m]E_m + 3K_a[C_m]^3E_t}{[C_t]} \quad (4)$$

The mean residue ellipticities of the monomeric and trimeric species and the coefficients A, B, C, and D were obtained by a simultaneous least-squares fit to eq 2, 3, and 4 by assuming that E_m and E_t are independent of temperature. It has been shown that this is a reasonable assumption for glucagon (Panijpan and Gratzer, 1974; Formisano et al., 1977).

The remaining thermodynamic parameters were evaluated from the appropriate derivatives of eq 2, ΔS° from eq 5, ΔH° from eq 6, and ΔC_p° from eq 7.

$$\Delta S^\circ = -\partial(\Delta G^\circ)/\partial T = -B - 2CT - 3DT^2 \quad (5)$$

$$\Delta H^\circ = \partial(\Delta G^\circ/T)/\partial(1/T) = A - CT^2 - 2DT^3 \quad (6)$$

$$\Delta C_p^\circ = \partial(\Delta H^\circ)/\partial T = -2CT - 6DT_2 \quad (7)$$

Sedimentation equilibrium measurements were performed with a Beckman Model E using the photoelectric scanner with 12-mm double-sector cells and a Beckman Model 3800 data recorder. This ultracentrifuge is equipped with a temperature control unit manufactured by Arden Instruments. The data were analyzed by nonlinear least-squares fitting to the exponential form of the concentration distribution, eq 1, 8, and 9:

$$[C_m] = [C_0]e^{\sigma(r^2/2-r_0^2/2)} \quad (8)$$

where

$$\sigma = \frac{M(1-\bar{v}\rho)\omega^2}{RT} \quad (9)$$

$[C_m]$ is the concentration of the monomer at any radius r, $[C_0]$ is the concentration of monomer at a reference radius r_0, ω is the angular velocity of the rotor, ρ is the density of the solution, R is the gas constant, T is the absolute temperature, M is the molecular weight of the monomer, and $\bar{v}$ is the partial specific volume.

The partial specific volume calculated from the amino acid composition is 0.71 mL/g (Cohn and Edsall, 1943). If the protein is not at its isoionic point the partial specific volume and the observed infinite dilution molecular weight must be corrected for the effects of the requirement of bulk electroneutrality throughout the solution. This is especially important when the charge of the protein is large, i.e., when the pH of the solution is far from the isoionic point of the protein. These effects have been treated by Yphantis and colleagues (Szuchet and Yphantis, 1973; 1976; Johnson and Yphantis, 1978). Calculations indicate that the apparent $\bar{v}$ of glucagon is lowered by about 0.03 mL/g, giving an effective $\bar{v}$ of 0.68 mL/g in this buffer.

"Best fits" were accomplished with an on line modeling program, MLAB, developed at NIH (Knott and Reese, 1972). This program utilizes the Marquardt–Levenberg algorithm to do nonlinear least-squares fit of data to an arbitrary equation (Marquardt, 1963). Reported standard errors of fitted parameters correspond to approximately 1 standard deviation.

Results

In our recent paper the thermodynamic parameters of glucagon association in 0.02 M K_2HPO_4, pH 10.6, were evaluated (Formisano et al., 1977). The ellipticity of glucagon at three concentrations as a function of temperature in 0.76 M phosphate, pH 10.6, is reported in Figure 1. The lines are theoretical and were determined by a simultaneous "best fit" to all of the data assuming a monomer–trimer equilibrium (eq 1, 3, and 4). The association constant, K_a, is a maximum at 286 °C in 0.76 M phosphate and equal to a free-energy change of -10.2 ± 0.2 kcal/mol. The thermodynamic parameters of association are shown in Figure 2 along with those obtained earlier in 0.20 M phosphate. The free energies of transfer (ΔG_t°) from 0.20 to 0.76 M phosphate are negative at all temperatures investigated. The negative free energies of transfer are derived completely from an increase in the entropy of transfer (ΔS_t°), since the enthalpy of transfer (ΔH_t°) is less favorable at all temperatures.

An independent measure of the self-association of glucagon was obtained from sedimentation equilibrium measurements, at 25 °C, 47 940 rpm, and 0.50 M phosphate. Data were collected at both 278 and 310 nm. By comparing the data from the two wavelengths, we were able to calculate an apparent ratio of extinction coefficients at 278 and 310 nm (the ratio in our instrument was 7.61). This ratio was used to evaluate

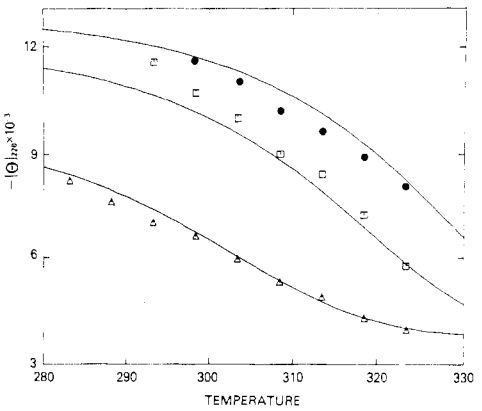

FIGURE 1: Mean residue ellipticity as a function of glucagon concentration and temperature in 0.76 M K_2HPO_4, pH 10.6. Protein concentrations were (●) 574, (□) 237, and (△) 63 μM. The "best fit" values of the monomer (E_m) and trimer (E_t) mean residue ellipticities are −3760 deg/mol and −14 090 deg/mol, respectively, The coefficients of eq 2 and 3 are (A) 28 850 cal/mol, (B) −141.8 cal deg^{-1} mol^{-1}, (C) −0.461 cal deg^{-2} mol^{-1}, and (D) 0.165 × 10^{-2} cal deg^{-2} mol^{-1}.

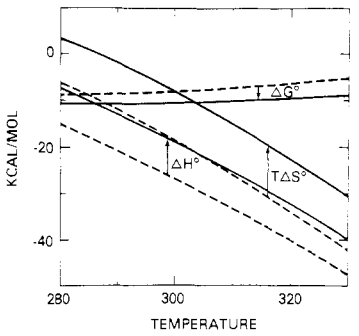

FIGURE 2: Thermodynamic parameters for the self-association of glucagon at pH 10.6. The solid lines correspond to the thermodynamic parameters at 0.76 M K_2HPO_4. The broken lines correspond to those previously reported at 0.20 M K_2HPO_4 (Formisano et al., 1977) and the arrows indicate the direction of the change in going from 0.20 to 0.76 M phosphate.

concentrations from the data at 310 nm. The data at both wavelengths for one of the cells are presented in Figure 3. This comparison of the two wavelengths enabled us to measure concentrations corresponding to optical densities as high as 7.0 at 278 nm. A simultaneous "best fit" of the absorption at 278 nm of three of the cells (at different loading concentrations) and two of the same cells at 310 nm gave a glucagon self-association constant, K_a, of 2.2 × 10^7 ± 0.9 × 10^7 M^{-2} and a molecular weight of 3680 ± 340, using a $\bar{v}$ of 0.68 mL/g. An alternative approach in the analysis of the sedimentation data is to specify the known monomer molecular weight, 3485, use 0.68 mL/g as $\bar{v}$, and calculate the association constant. When this approach was used, the association constant, K_a, was found to be 3.2 × 10^7 ± 0.3 × 10^7 M^{-2}. In either case the average deviation between the fitted line and the experimental data points is approximately 0.02 OD, the approximate resolution of our instrument. The best value of the association constant, K_a, at 0.5 M phosphate should be taken as 2.2 × 10^7 ± 0.9 × 10^7 M^{-2}, since this was determined without assuming a value of $\bar{v}$. This equilibrium constant is consistent with the values obtained from CD.

The effect of the phosphate, fluoride, and chloride salts of potassium on the mean residue ellipticity of glucagon at pH 10.6 is shown in Figure 4. Plateau values of the ellipticity could not be attained due to the insolubility of glucagon at higher concentrations of these salts.

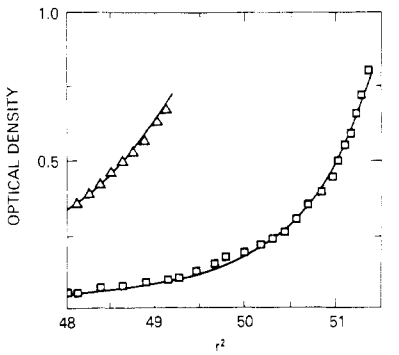

FIGURE 3: Comparison of the optical densities at 278 (△) and 310 nm (□) for the sedimentation equilibrium experiment described in the text. Solid lines correspond to a monomer molecular weight of 3680 and a trimerization constant of 2.2 × 10^7 M^{-2}. See text for details.

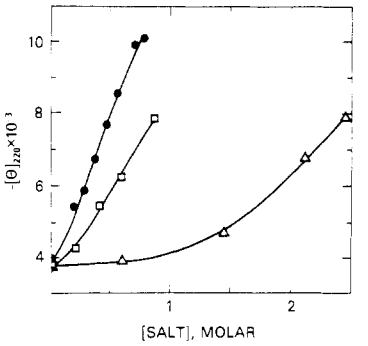

FIGURE 4: Mean residue ellipticity of glucagon at 220 nm as a function of salt concentration; K_2HPO_4 (●), KF (□), and KCl (△). Glucagon concentration is 150 μM in 9 mM K_2HPO_4 buffer, pH 10.6, and 25 °C. The high absorption with KBr, KNO_3, KSCN, and potassium acetate prevented the measurement of the ellipticity at 220 nm. Solid lines are calculated as described in the text.

Klotz (1966) and Tanford (1969) have pointed out that any third component can modify the conformation (or association) equilibrium of a protein either by preferentially binding to one of the equilibrium species of the protein or by changing the activity of the water. From the Gibbs–Duhem equation it has been shown:

$$\frac{d \ln K_a}{d \ln a_3} = \Delta\bar{\nu}_3 - \left(\frac{n_3}{n_1}\right)\Delta\bar{\nu}_1 \qquad (10)$$

where component 3 represents the salt, 1 the water, and 2 the protein. $\Delta\bar{\nu}_3$ and $\Delta\bar{\nu}_1$ are the changes in the number of moles bound, while n_3 and n_1 are the number of moles of each component present in solution, respectively.

The ellipticity data presented in Figure 4 can be analyzed according to eq 10, by a "best fit" to eq 1 and 4, where K_a is expressed as the integral of eq 10. By using the values of the monomer and trimer ellipticities, E_m and E_t, determined previously, in Figure 1, the problem is reduced to a three-parameter curve fit, namely, $\Delta\bar{\nu}_1$, $\Delta\bar{\nu}_3$, and an integration constant. The solid lines in Figure 4 correspond to the "best fit" of the data.

The sensitivities of the "best fit" for the parameters of eq 10 were evaluated by fixing a particular parameter and then determining a "best fit" of the remaining parameters. The variances of these fits as a function of the parameters are a measure of the precision of the curve-fitting precedure. Figure 5 presents an analysis of this type for the evaluation of $\Delta\bar{\nu}_3$ and $\Delta\bar{\nu}_1$ from the data in Figure 4. There was little change in $\Delta\bar{\nu}_3$ for the three salts, the minimum varying between 0.5 and 1.5 mol of salt per mol of trimer. However, there is a systematic

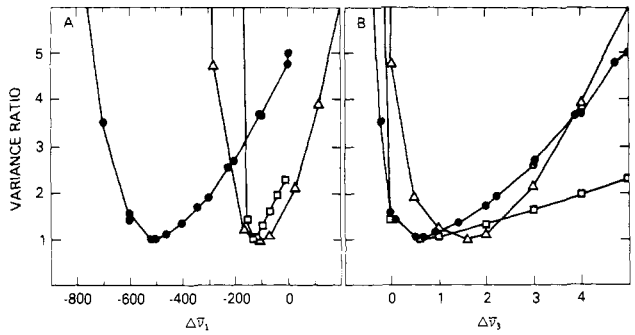

FIGURE 5: Analysis of the difference in the number of water molecules (A) and the number of the salt molecules (B) bound in the self-association. The symbols refer to K_2HPO_4 (●), KF (□), and KCl (△). See text for details.

decrease in the number of water molecules bound to the trimer. If $\Delta\bar{\nu}_3$ is fixed at an average value of 1 mol per mol of trimer, the values of $\Delta\bar{\nu}_1$ for the different salts are: -461 ± 27 for K_2HPO_4, -166 ± 17 for KF, and -123 ± 6 for KCl per mol of trimeric glucagon. The negative signs correspond to water which is released from three glucagon monomers when they associate to form a trimer. Unfortunately, unequivocal values of $\Delta\bar{\nu}_3$ and $\Delta\bar{\nu}_1$ cannot be found due to the high correlation between these two parameters. These data are consistent enough to indicate that the effects of the various salts cannot be described solely as a salt-binding phenomena ($\Delta\bar{\nu}_1 = 0$ in Figure 5A). The data can be fit, to a model where the sole effect is due to the change in the activity of water ($\Delta\bar{\nu}_3 = 0$ in Figure 5B). However, a better fit is obtained when both mechanisms are operative, i.e., the binding of approximately one molecule of salt to the trimer and a systematic decrease in the number of water molecules bound to the trimer compared to the monomer for the series K_2HPO_4, KF, and KCl. Similar results have been obtained by Aune et al. (1971) on the effect of NaCl and $CaCl_2$ on the dimerization of α-chymotrypsin. The preferential binding of water to glyceraldehyde-3-phosphate dehydrogenase has been shown to explain the decrease in sedimentation rate with phosphate (Aune and Timasheff, 1970).

It is to be expected that the formation of a peptide hydrogen bond will release the water of hydration of the C=O and N-H groups in the peptide backbone. Since there is a large increase in the α-helical content with trimer formation, a large amount of water should be released with association. The water in the "icelike" shell around the nonpolar moieties will also become more like bulk water when the nonpolar groups interact with each other. Therefore, when glucagon forms a trimer there are at least two mechanisms by which water molecules are removed from contact with monomers.

TABLE I: The Effects of Various Salts on the Thermodynamic Transfer Parameters of the Peptide and Methylene Groups in Water.[a]

	T (°C)	$\Delta G_t°$ (kcal/mol)	$\Delta H_t°$ (kcal/mol)	$T\Delta S_t°$ (kcal/mol)
		Peptide Group		
Na_2SO_4	0.5	−0.040	−0.39	−0.34
	40.0	+0.010		−0.40
NaCl	0.5	−0.100	−0.45	−0.35
	40.0	−0.050		−0.40
NaSCN	0.5	−0.190	−0.78	−0.59
	40.0	−0.105		−0.67
$NaClO_4$	0.5	−0.235	−1.03	−0.80
	40.0	−0.120		−0.91
KCl	0.5	−0.090	−0.44	−0.35
	40.0	−0.040		−0.40
LiCl	0.5	−0.105	−0.52	−0.42
	40.0	−0.045		−0.48
		CH_2 Group		
Na_2SO_4	0.5	0.310	1.07	0.76
	40.0	0.200		0.87
NaCl	0.5	0.115	0.36	0.24
	40.0	0.080		0.28
KCl	0.5	0.090	0.16	0.07
	40.0	0.080		0.08

[a] All thermodynamic parameters are for the transfer from water to 2 M salt except Na_2SO_4 which is for 1 M. We have therefore doubled the values for Na_2SO_4. Calculated as described in the text from the data of Nandi and Robinson (1972a,b).

Discussion

The Hofmeister anions change the solubility and stability of proteins by their salting-out of nonpolar groups and salting-in of peptide groups. Although considerable free-energy data are available on the effects of the Hofmeister anions, very few studies exist in which the enthalpic and entropic contributions to the free-energy changes were evaluated.

Glucagon offers an interesting model for evaluating neutral salt effects, since the self-association not only involves intersubunit contacts between hydrophobic side chains but also a large increase in hydrogen bonding, i.e., α-helical residues. Thus, the formation of secondary and tertiary structure with association resembles the conformational changes which take place when a nascent polypeptide chain is released from polyribosomes and folds into its native globular structure. In fact, the formation of globular proteins such as ribonuclease (Brandts and Hunt, 1967), chymotrypsinogen (Brandts, 1964a,b), etc. (as measured by denaturation) and glucagon self-association have negative enthalpies and entropies which decrease with increasing temperature. Since hydrophobic reactions are usually considered to be entropically driven, the above reactions appear anomalous or controlled by other interactions (Kauzmann, 1959). However, if one takes into account the large change in α-helical hydrogen bonding as well, the association can be enthalpically driven and still controlled by hydrophobic interactions, as shown by the work of Scheraga and colleagues (for example, von Dreele et al., 1971; Alter et al., 1972; Maxfield et al., 1975). In an extensive series of articles they have reported that many nonpolar residues gave negative enthalpy changes when participating in a coil → α-helix transition. Most of the guest residues they investigated in their host–guest analysis of the thermal transitions in either poly(hydroxybutylglutamine) or poly(hydroxypropylglutamine) gave negative enthalpy changes. In the direct transfer of nonpolar groups from water to organic solvents, however, the enthalpy change is normally positive, at least below 40 °C (Kauzmann, 1959; Brandts, 1964a,b; Edelhoch and Osborne, 1976). In the latter case, the negative free-energy change of transfer is due to the very favorable entropy of transfer.

Increasing phosphate from 0.20 to 0.76 M decreases the free energy and increases the entropy and enthalpy of glucagon association. Thus, although the association is still enthalpically driven, it is the increase in the entropy of transfer which is responsible for the greater association in 0.76 M phoshpate.

It has been known for a long time that anions salt-out nonpolar molecules with the Hofmeister ranking. It has only recently been shown that the peptide group is salted-in by most of the Hofmeister anions (Schrier and Schrier, 1967; Nandi and Robinson, 1972a,b). The Hofmeister specificity depends

TABLE II: The Effect of NaCl on the Thermodynamic Transfer Parameters of Methane and Ethane from Water to 1 M NaCl.[a]

Temp (°C)	$\Delta G_t°$ (cal/mol)	$\Delta H_t°$ (cal/mol)	$T\Delta S_t°$ (cal/mol)	$\Delta C_p°$ (cal/mol-deg)
		Methane		
10	209	463	254	1.2
15	204	469	264	1.3
20	200	475	276	1.3
25	195	482	287	1.4
30	190	489	299	1.4
		Ethane		
10	248	485	237	12.6
15	243	549	306	13.1
20	237	616	379	13.7
25	230	686	456	14.3
30	222	759	537	14.9

[a] Calculated from data in Ben-Naim and Yaacobi (1974). It should be noted that these data are in terms of Ostwald coefficients. These have been explicitly defined by Ben-Naim (1974).

on the interaction between the anions and the nonpolar groups (von Hippel and Hamabata, 1973).

Only a few studies are available of the effect of temperature on anion interactions with small polar or nonpolar molecules. Nandi and Robinson (1972a,b) have determined the free-energy changes from solubility studies for the transfer of both the CH_2 and peptide groups from water to 2 M solutions of several Hofmeister salts at 0.5 and 40.0 °C. We have calculated the enthalpy and entropy changes from their data (Table I). The effect of three salts on the thermodynamic constants of the CH_2 groups shows that the enthalpy and entropy changes of transfer are positive and $\Delta H_t° > T\Delta S_t°$. The values of each parameter are greater for sulfate than chloride.

The enthalpy and entropy changes of transfer for the peptide group in small polypeptides are both negative and $|\Delta H_t°| > |T\Delta S_t°|$. Both values decrease with the salting-in effectiveness of the Hofmeister series, perchlorate being greater than sulfate. These parameters are based on solubility studies of various low-molecular-weight amino acids and small polypeptides and, consequently, refer to the interaction of functional groups with water, i.e., their exposure to water. In order to compare these values to those of glucagon, we must compare them with glucagon dissociation, i.e., the exposure of groups which are not exposed to water in the associated state. The transfer parameters are considered in the direction of increasing concentration, from 0 to 2 M for the model compounds and from 0.20 to 0.76 M phosphate for glucagon.

The negative signs of the transfer enthalpy and entropy changes for glucagon dissociation are in accord with those of the peptide group and not with those of the CH_2 group. However, the free energy of transfer is positive and agrees with that of the CH_2 group transfer values. The positive free energy of transfer observed for glucagon dissociation can be explained by including a contribution from the hydrophobic interactions, since $\Delta H_t° > T\Delta S_t°$ for the CH_2 group. In glucagon the predominant contribution to $\Delta H_t°$ and $T\Delta S_t°$ comes from the peptide groups rather than hydrophobic groups, presumably due to the very large decrease in α-helical content with dissociation. We have seen that the dissociation of glucagon leads to the binding of a rather large number of water molecules for all three salts investigated. In other protein reactions involving principally exposure of the nonpolar groups, the effects of Hofmeister salts could be reversed, i.e., positive values of $\Delta H_t°$ and $T\Delta S_t°$.

We have also analyzed the solubility data of Ben-Naim and Yaacobi (1974) for CH_4 and C_2H_6 in water and NaCl solutions of 0.25, 0.50, 1.00 and 2.00 M between 10 and 30 °C at 5 °C intervals. We first determined the dependence of the Ostwald absorption coefficients on NaCl for each of the temperatures by a linear least-squares analysis to obtain the transfer free energy of the gases from water to 1 M NaCl (Table II). From these values we calculated the enthalpy, entropy, and heat-capacity changes (Table II). All the transfer parameters are positive, in accord with the data of Nandi and Robinson for the CH_2 in NaCl solution. However, the data for ethane reveal a linear change of $\Delta H_t°$ with temperature and a heat capacity of transfer of approximately 13 cal/mol-deg. Since the enthalpies of transfer of methane are only slightly dependent on temperature, the difference in the enthalpies of transfer between CH_4 and C_2H_6 depends strongly on temperature. Since Nandi and Robinson collected their data only at two temperatures, i.e., 0.5 and 40 °C, we do not know if the enthalpies of transfer of the CH_2 and peptide groups are temperature dependent.

TABLE III: The Thermodynamic Parameters of the α-Helix → Coil Transition of the 1-13 N-Terminal Fragment of Ribonuclease.[a]

Molar concn	T (°C)	α	$\Delta G°$ (kcal/mol)	$\Delta H°$ (kcal/mol)	$T\Delta S°$ (kcal/mol)
			Na_2SO_4		
0.12	1	0.45	−0.11	5.7	5.8
	26	0.24	−0.64		6.4
0.33	1	0.73	+0.58	12.1	11.6
	26	0.29	−0.48		12.6
0.78	1	0.91	+1.40	17.0	15.8
	26	0.48	−0.04		17.2
			$(NH_4)_2SO_4$		
0.12	1	0.43	−0.16	6.8	7.0
	26	0.18	−0.80		7.6
0.33	1	0.59	+0.20	9.6	9.4
	26	0.23	−0.65		10.2
1.00	1	0.81	+0.84	12.5	11.6
	26	0.40	−0.22		12.7

[a] Calculated as described in the text from the data on Brown and Klee (1971). α = fraction α-helix. $\Delta G° = -RT \ln$ (coil/helix). Since the ellipticity value of the α-helix species is only approximate, the errors in the thermodynamic parameters will increase significantly as α approaches zero or unity.

Klee (1968; Brown and Klee, 1969; 1971) has evaluated the effect of Na_2SO_4 (in water and 0.033 M) on the mean residue ellipticities of four polypeptides isolated from the N-terminus of ribonuclease: residues 1–8, 1–13, 1–15, and 1–20. Residues 2–12 are α-helical in the enzyme. The three longer chains show transitions whose equilibria depend on the ionic strength and temperature. The 1–8 chain is devoid of α-helical residues under the same conditions.

The ellipticities of the 1–13 polypeptide were determined as a function of ionic strength for Na_2SO_4 and $(NH_4)_2SO_4$ at 1 and 26 °C. We have calculated the equilibrium constant for the α-helix $\rightarrow$ coil transition at the three highest ionic strengths at both temperatures (Table III) from the ellipticity values. The equilibrium constants are given for the α-helix $\rightarrow$ coil transition in order to keep the direction of the change consistent with that of the solubility data, i.e., exposure of groups to water. The value used for the coil was that found in Brown and Klee (1971) in 5 M guanidine hydrochloride, +1000 deg/mol, while that used for the α-helix was that found in 90% trifluoroethanol, −11 500 deg/mol. The coil value is reasonable, since similar values (±1000) were obtained for the 1–8 polypeptide chain in water and 0.033 M Na_2SO_4 at 1 and 26 °C where there is no evidence of any organized structure. Moreover, there is not likely to be any α-helical structure in a molecule this small containing polar and charged groups. The value used for the α-helix is an upper limit but must be approximately correct, since a value of −10 500 deg/mol was reached at the highest ionic strength and the curve showing the dependence of ellipticity on ionic strength is still increasing. In view of the uncertainty in the two limiting ellipticity values, the equilibrium constants and thermodynamic parameters should only be considered as approximate.

The thermodynamic parameters are reported in Table III for the three highest ionic strengths studied. At lower ionic strength, 0.1 to water, there is only a very minor, monotonic change in ellipticity (Figure 4 of Brown and Klee, 1971). Consequently, it appears that the major influence of ionic strength on conformation is related to Hofmeister effects and not Debye–Hückel effects. The transfer enthalpy and entropy changes are positive and increase with increasing ionic strength (Table III). This result implicates hydrophobic interactions as being responsible for the shift of the coil into the α-helical form with increasing salt. The signs of the two parameters are opposite from that of glucagon dissociation. One explanation of the change from a largely peptide-driven reaction in glucagon to a nonpolar one in the 1–13 fragment is that the maximum extent of α-helical formation is much smaller in the 1–13 fragment than in glucagon because the smaller polypeptide cannot form more than a 1–2 turn α-helix (Goodman et al., 1962). This limits the number of peptide groups which release water molecules on forming the helical structure.

It thus appears that the sulfate and phosphate anions, which are located on the salting-out end of the Hofmeister series, can fold and associate proteins either through their influence on hydrophobic interactions or on the α-helix $\rightarrow$ coil transition. In either case, the net effect is to enhance structure formation with increasing salt concentration.

References

Alter, J. E., Taylor, G. T., and Scheraga, H. A. (1972), *Macromolecules 5,* 739–746.

Aune, K. C., Goldsmith, L. C., and Timasheff, S. N. (1971), *Biochemistry 10,* 1617–1622.

Aune, K. C., and Timasheff, S. N. (1970), *Biochemistry 9,* 1481–1484.

Ben-Naim, A. (1974), Water and Aqueous Solutions, Introduction to a Molecular Theory, New York, N.Y., Plenum.

Ben-Naim, A., and Yaacobi, M. (1974), *J. Phys. Chem. 78,* 170–175.

Blanchard, M. H., and King, M. W. (1966), *Biochem. Biophys. Res. Commun. 25,* 298–303.

Brandts, J. F. (1964a), *J. Am. Chem. Soc. 86,* 4291–4301.

Brandts, J. F. (1964b), *J. Am. Chem. Soc. 86,* 4302–4314.

Brandts, J. F., and Hunt, L. (1967), *J. Am. Chem. Soc. 89,* 4826–4838.

Brown, J. E., and Klee, W. A. (1969), *Biochemistry 8,* 2876–2879.

Brown, J. E., and Klee, W. A. (1971), *Biochemistry 10,* 470–476.

Cohn, E. J., and Edsell, J. T. (1943), Proteins, Amino Acids and Peptides, New York, N.Y., Reinhold, p 375.

Edelhoch, H., and Osborne, J. C. (1976), *Adv. Protein Chem. 30,* 183–250.

Formisano, S., Johnson, M. L., and Edelhoch, H. (1977), *Proc. Natl. Acad. Sci. U.S.A. 74,* 3340–3344.

Goodman, M., Schmitt, E. E., and Yphantis, D. A. (1962), *J. Am. Chem. Soc. 84,* 1288–1296.

Gratzer, W. B., and Beaven, G. H. (1969), *J. Biol. Chem. 244,* 6675–6679.

Gratzer, W. B., Creeth, J. M., and Beaven, G. H. (1972), *Eur. J. Biochem. 31,* 505–509.

Johnson, M. L., and Yphantis, D. A. (1978) *Biochemistry* (in press).

Kauzmann, W. (1959), *Adv. Protein Chem. 14,* 1–63.

Klee, W. A. (1968), *Biochemistry 7,* 2731–2736.

Klotz, I. M. (1966), *Arch. Biochem. Biophys. 116,* 92–96.

Knott, G. D., and Reese, D. K. (1972), *Proc. ONLINE '72 Int. Conf. 1,* 497–526.

Marquardt, D. W. (1963), *J. SIAM 11,* 431–441.

Maxfield, F. R., Alter, J. E., Taylor, G. T., and Scheraga, H. A. (1975), *Macromolecules 8,* 479–491.

Nandi, P. K., and Robinson, D. R. (1972a), *J. Am. Chem. Soc. 94,* 1299–1308.

Nandi, P. K., and Robinson, D. R. (1972b), *J. Am. Chem. Soc. 94,* 1308–1315.

Panijpan, B., and Gratzer, W. B. (1974), *Eur. J. Biochem. 45,* 547–533.

Sasaki, K., Dockerill, S., Adamiak, D. A., Tickle, I. J., and Blundell, T. (1975), *Nature (London) 257,* 751–757.

Schrier, E. E., and Schrier, E. B. (1967), *J. Phys. Chem. 71,* 1851–1860.

Szuchet, S., and Yphantis, D. A. (1973), *Biochemistry 12,* 5115–5127.

Szuchet, S., and Yphantis, D. A. (1976), *Arch. Biochem. Biophys. 173,* 495–516.

Tanford, C. (1969), *J. Mol. Biol. 39,* 539–544.

von Dreele, P. H., Lotan, N., Ananthanarayanan, V. S., Andreatta, R. H., Poland, D., and Scheraga, H. A. (1971), *Macromolecules 4,* 408–417.

von Hippel, P. H., and Hamabata, A. (1973), *J. Mechanochem. Cell Motil. 2,* 127–138.

von Hippel, R. H., and Schleich, T. (1969), in Structure and Stability of Biological Macromolecules, Timasheff, S. N., and Gasman, G. D., Ed, New York, N.Y., Marcel Dekker, p 417.