Table VII. Comparison of Solubilities and ΔF_{tr} for ATGEE at 25°, Based on the Molarity and Mole Fraction Scales

Solvent	M	Molarity scale S/S^0	Molarity scale $\Delta F_{tr,S}$,[a] cal./mole	Mole fraction scale N/N^0	Mole fraction scale $\Delta F_{tr,N}$,[a] cal./mole
Urea	3	1.85	−366	2.0	−410
Urea	8	3.3	−710	4.15	−850
1,3-Dimethylurea	3	1.41	−206	1.7	−315
Guanidine hydrochloride	3	3.5	−745	4.7	−920
Guanidine hydrochloride	7	7.4	−1190	12	−1480
Tetramethylguanidine hydrochloride	3	0.63	+266	0.96	+27
Formamide	3	1.25	−150	1.32	−165
N,N-Dimethylformamide	3	0.89	−70	1.06	−35
Ethanol	3	0.85	+96	0.96	+27
Dioxane	3	0.98	+14	1.17	−96

[a] $\Delta F_{tr,S} = RT \ln S^0/S$; $\Delta F_{tr,N} = RT \ln N^0/N$; ΔF_{tr} represents the free energy of transfer of solute from water to another solvent at the same solute concentration.

larger than the value of −145 cal./mole, which was calculated from our data by Nozaki and Tanford for the contribution of a glycyl residue in this process.[8] However, the calculation of the latter value was based upon the assumptions that the acetyl and ester groups of ATGEE have the same effect on the free energy of transfer as butane and that additivity of free energy contributions holds for individual adjacent peptide units.

The same values, −710 and −1190 cal./mole, respectively, are obtained if the interaction of ATGEE with urea or guanidine hydrochloride is treated as a direct binding and the free energies of transfer are calculated from eq. 4[22b] and the equilibrium constants of Table V.

$$\Delta F = -nR + \ln (1 + K[\text{denaturant}]) \qquad (4)$$

This treatment differs from that of Schellman in that a single binding site[5] is assumed for each peptide molecule, rather than separate sites for each NH and CO group.[22b]

In our opinion, more data is required before numbers of this kind can be used for a detailed calculation of the free energy of protein denaturation in different solvents. The principal problems are the absence of additivity of the free energy contributions of adjacent glycyl units and the absence of data on the effects of side chains on the denaturant–peptide interaction. Studies of the effects of urea and guanidine hydrochloride on more complex, short, uncharged peptides may provide further information on these questions.

The Effect of Concentrated Salt Solutions on the Activity Coefficient of Acetyltetraglycine Ethyl Ester

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Contribution No. 354 from the Graduate Department of Biochemistry, Brandeis University, Waltham, Massachusetts. *Received December 31, 1964*

The activity coefficient of the uncharged model peptide, acetyltetraglycine ethyl ester (ATGEE), has been determined in the presence of concentrated salt solutions by solubility measurements. The results suggest that the effects of concentrated salt solutions on the denaturation, dissociation, and solubility of proteins may be accounted for, in large part, by effects of the salts on the peptide and amide groups which become exposed to the solvent during these processes. The results cannot easily be explained by effects of the salt solutions on the availability of solvating water, by effects on water "structure," by electrostatic treatments of the Debye–Kirkwood type, or by effects on the internal pressure of the solvent. The results are consistent with a summation of two effects: (1) an ordinary "salting-out" effect, which may be described in terms of the average cohesive energy or internal pressure of the solvent, and (2) a direct interaction between certain large anions and the amide dipole. The second of these interpretations is supported by the close correlation between the order of effectiveness of anions toward ATGEE and their order of binding to anion-exchange resins and other charged groups. In addition, the results provide evidence for the existence, in aqueous solution, of an interaction between aromatic and other highly polarizable compounds and the amide group, which may be of importance in maintaining protein structure and in protein–solvent and protein–solute interactions.

Introduction

The Hofmeister or lyotropic series of ions, which describes the order of effectiveness of ions in influencing a very large number of chemical and physical phenomena, was determined by Hofmeister by measurements of the relative effectiveness of ions in causing the precipitation of proteins.[1,2] With minor exceptions, the same order of activity of ions is found for denaturation, depolymerization, and dissociation of proteins and for the inhibition or activation of a number of enzymes. In general, those ions which are most effective in causing protein precipitation also are most effective in preventing denaturation and dissociation into subunits, and those ions which increase the solubility of proteins

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(2) (a) A. Voet, *Chem. Rev.*, 20, 169 (1937); (b) J. W. McBain, "Colloid Science," Reinhold Publishing Corp., New York, N. Y., 1950, Chapter 9.

favor denaturation and dissociation of proteins.[1,3-6] In spite of an extensive literature on the effects of concentrated salt solutions on the structure of water,[7-9] and the salting out of organic solutes,[10] the mechanism of the effects of such salt solutions on the properties of proteins has not been determined. Bello, Riese, and Vinograd showed that the effect of salts on the melting of gelatin gels, which may be regarded as a form of protein denaturation and follows the same order of sensitivity to salts, does not result from interactions of the salts with the charged groups of the protein, and suggested the existence of a direct interaction of the ions with the peptide backbone.[11] Meyer and Klemm showed that the solubility of diketopiperazine is increased by concentrated solutions of alkali bromides and iodides and suggested that a direct ion–amide interaction accounts for the increased solubility of this compound and of proteins in the presence of these salts.[12] After the completion of the work reported here, von Hippel and Wong described the effects of ions on the denaturation of ribonuclease and on other properties of proteins in a manner somewhat similar to that adopted here, but reached different conclusions.[5] In an attempt to obtain information bearing on these questions, we have determined the effect of a number of salt solutions on the solubility and activity coefficient of acetyltetraglycine ethyl ester, ATGEE, a model for the peptide and amide groups of proteins. The results demonstrate that a large part of the effect of concentrated salt solutions on proteins may be accounted for by their effects on the peptide and amide groups which become exposed to the solvent when the protein undergoes a change in physical state. Although the mechanism of these effects has not been established, the results provide evidence against a number of proposed mechanisms and are consistent with a combination of two mechanisms.

Experimental

Materials and methods were essentially as described in the preceding paper.[13] Inorganic salts were reagent grade materials and were used without further purification. Organic salts were recrystallized before use. Phenol was purified by sublimation. Sodium perchlorate, lithium 3,5-diiodosalicylate, sodium benzoate, sodium acetate, and sodium trichloroacetate were prepared by neutralizing the corresponding acids.

The concentrations of halide salts were determined by Volhard silver nitrate titration and thiocyanate concentration was determined by a modification of this procedure.[14] Phosphate was determined by the Fiske–Subbarow method.[15] Solutions of sodium nitrate, sodium thiosulfate, sodium bromate, and potassium fluoride were prepared by diluting saturated solutions at 25°. The concentrations of saturated solutions were taken from the literature.[16] The concentrations of sodium trichloroacetate, sodium acetate, and sodium perchlorate solutions were known from the amount of base required to neutralize the respective acids. Sodium sulfate, sodium tosylate, benzoic acid, glycine, and sodium bisulfite solutions were prepared gravimetrically. Phenol concentration was determined by ultraviolet spectrophotometry. Solutions containing iodide were stabilized with approximately 5×10^{-4} *M* sodium thiosulfate. Glass-distilled water was used throughout.

The attainment of equilibrium in the solubility determinations was demonstrated for most of the salts by one of the two procedures described previously.[13] A few determinations were made with a single measurement after 72 hr. of equilibration at 25°, after it had been established that equilibrium was reached within 72 hr. at 25° with a large number of salts. Equilibrations at 0 and 40° were carried out for 7 days and 24 hr., respectively, and it was shown that equilibrium was reached in each case.

Solutions were adjusted to a pH value between 3 and 7. More alkaline or acid conditions resulted in a slow increase of biuret-reacting material with time, presumably because of hydrolysis of ATGEE. Solutions of potassium fluoride were found to become more alkaline with time. A 1 *M* solution of potassium fluoride was found to have a pH of 8.0, which may have given a value of the activity coefficient which is low by as much as 15% at this concentration; in 0.5 and 0.25 *M* potassium fluoride solutions the pH was not sufficiently high to cause significant errors.

The concentration of ATGEE was generally determined by the biuret method.[17] Standard solutions of biuret or ATGEE were assayed in the presence of each salt, and no significant interference was noted after subtracting appropriate blanks. The concentration of ATGEE in the presence of divalent cations was determined by measurements of the absorption of the amide group in the far-ultraviolet. The difference in the absorbance at 225 and 250 mμ was measured and was found to be proportional to the concentration of ATGEE over the range of concentration of these experiments. The salt solutions were found to have no appreciable effect on the absorbance of a standard solution of ATGEE at these wave lengths. Spectrophotometric measurements were made with a Zeiss PMQ II spectrophotometer, utilizing a light path of 0.2 to 5 cm. Measurements of pH were made with Radiometer PHM 4 and PHM 22 pH meters.

Results

The effects of salt solutions on the activity coefficient of ATGEE were determined by solubility measurements, as described in the preceding paper. The

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(17) A. G. Gornall, C. J. Bardawill, and M. M. David, *J. Biol. Chem.*, **177**, 751 (1949).

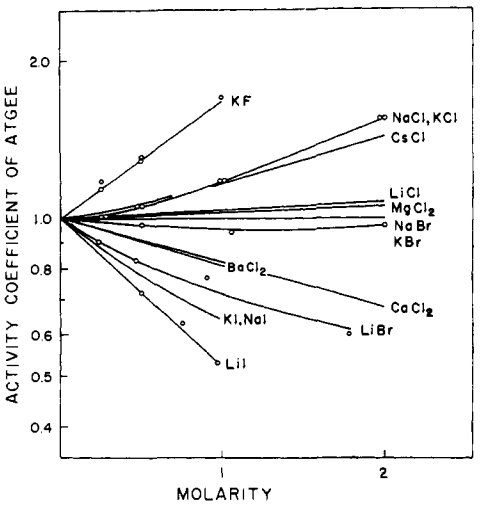

Figure 1. Activity coefficient of ATGEE in solutions of halide salts at 25.0°, determined by solubility measurements.

results for a series of alkali and alkaline earth halides are shown in Figure 1 and for a larger series of ions of different structures in Figure 2. For clarity of presentation, experimental points are not shown for all of the salts, but the points which are shown are representative of the accuracy of the data obtained for all salts. The semilogarithmic plots of activity coefficient against salt concentration are linear for a number of the salts which, therefore, follow the Setschenow equation (1)

$$\log \gamma = \log S_0/S = K_s C_s \qquad (1)$$

in which γ is the activity coefficient of ATGEE in the salt solution, S_0 and S are the molar solubility of ATGEE in water and the salt solution, respectively, K_s is the salting-out constant, and C_s is the molar concentration of salt. The initial slopes, K_s, were calculated for all of the salts which were examined and are summarized in Table I. The K_s values are approximate for many of the salts, because of curvature of the plots. They are, nevertheless, useful for comparing the effects of different salts on the activity coefficient of ATGEE. With the exception of sodium trichloroacetate, the order of the effectiveness of those salts which were examined at concentrations of 1 or 2 M does not differ significantly from the order of the K_s values. The results obtained with phenol are included in Figure 2 for comparison with the effects of salts which contain aromatic groups. Quinoxaline, another uncharged aromatic compound, was found to cause strong salting in at 0.25 M, but no further change was observed at 0.5, 1.0, and 2.0 M, possibly because of the formation of an insoluble complex with the peptide.

Separately, 3 M urea and 0.5 M sodium sulfate cause salting in and salting out with $S°/S$ values of 0.54 and 1.92, respectively. A solution of 0.5 M sodium sulfate in 3 M urea was found to cause neither salting in nor salting out (K_s = 1.04), which indicates that the salting out effects of these two compounds are approximately additive.

Effect of Temperature. The solubility of ATGEE in sodium iodide solutions at 0, 25, and 40° is shown in Figure 3. The effect of sodium iodide increases with decreasing temperature over the range 0–40°. The activity coefficient is linear with respect to salt concentration at all three temperatures, and remains linear up to 3.3 M sodium iodide at 25°.

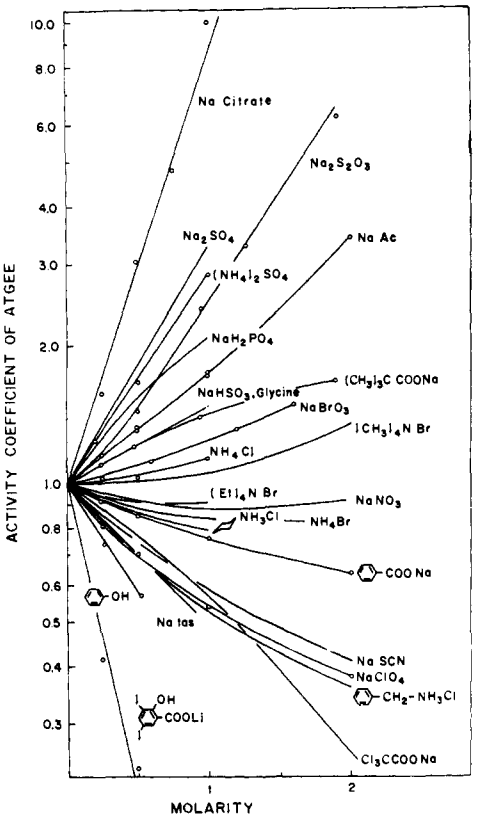

Figure 2. Activity coefficients of ATGEE in solutions of various salts and phenol at 25.0°.

Discussion

Effects of Salts on ATGEE. The following general statements can be made regarding the effects of salts on the activity coefficient of ATGEE.

(1) In general, the differences between different salts reflect principally differences in the effects of the

Table I. Approximate Salting-Out Constants for Acetyltetraglycine Ethyl Ester at 25.0°

Compd.	K_s[a]	Compd.	K_s[a]
Lithium 3,5-diiodosalicylate	−1.3	NaBr	0.00
Phenol	−0.48	$MgCl_2$	0.00
$NaClO_4$	−0.33	$(CH_3)_4NBr$	+0.018
Sodium tosylate	−0.31	LiCl	+0.021
$C_6H_5NH_3Cl$	−0.31	NH_4Cl	+0.035
LiI	−0.28	KCl	+0.046
$Cl_3CCOONa$	−0.27	NaCl	+0.046
NaSCN	−0.25	CsCl	+0.054
NaI	−0.23	$NaBrO_3$	+0.090
KI	−0.21	$(CH_3)_3CCOONa$	+0.15
LiBr	−0.17	Glycine	+0.16
C_6H_5COONa	−0.14	$NaHSO_3$	+0.16
$C_6H_{11}NH_3Cl$	−0.13	KF	+0.23
NH_4Br	−0.11	CH_3COONa	+0.23
$(CH_3CH_2)_4NBr$	−0.11	$Na_2S_2O_3$	+0.35
$BaCl_2$	−0.11	NaH_2PO_4	+0.36
$CaCl_2$	−0.09	$(NH_4)_2SO_4$	+0.45
$NaNO_3$	−0.075	Na_2SO_4	+0.48
KBr	−0.023	Na_3 citrate	+0.90

[a] Log $S°/S = K_sM$, where $S°$ is the solubility in water, S is the solubility in other solvent, and M is the concentration of salt in moles per liter. Values of K_s were estimated from solubility measurements at salt concentration 0–0.5 M.

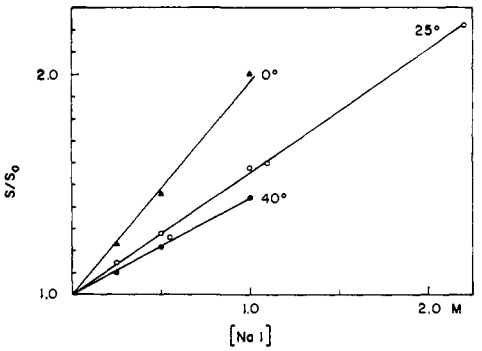

Figure 3. Effect of temperature on the solubility of ATGEE in solutions of sodium iodide.

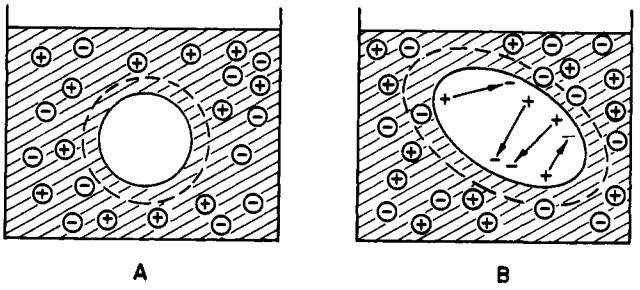

Figure 4. Diagram illustrating the ion-free area surrounding a nonpolar solute in an aqueous solution containing small ions (A) and the approach of large anions to the positive ends of dipoles of the polar solute (B).

anions. In the series of alkali halides (Figure 1), fluoride salts out strongly, chloride salts out less strongly, bromide has little effect, and iodide salts in strongly. A similar large sensitivity to the nature of the anion, which generally follows the Hofmeister series, is found with the other salts examined. Variation in the nature of the monovalent cation has a much smaller effect. The halides of Na^+, K^+, NH_4^+, Cs^+, and $(CH_3)_4N^+$ all have nearly the same effects. Salts of lithium and divalent cations exhibit a tendency toward salting in, compared to salts of other cations. The only cation which is associated with a large salting-in effect is benzylammonium chloride, which contains an aromatic group. The results with the alkali halides are very similar to those observed by Meyer and Klemm with diketopiperazine in the presence of high salt concentrations, but do not show the pronounced nonlinearity observed by these authors at low concentrations of certain salts.[12]

(2) The ions exert their effects independently, *i.e.*, the relative effects of Cl^-, Br^-, and I^- are similar, regardless of the nature of the alkali cation, even for a cation, such as lithium, which has a different effect from that of other cations.

(3) The presence of an aromatic group in either the anion or cation greatly enhances salting in. This is illustrated by the salting in by sodium benzoate compared to salting out by sodium acetate, the strong salting in by benzylammonium chloride compared to the much weaker effect of cyclohexylammonium chloride, the strong salting in by sodium tosylate compared to the salting out by bisulfite, and the very large salting-in effect of diiodosalicylate. The effect of phenol was examined in order to determine the effect of an aromatic compound in the absence of a charged group and it was found that phenol has a strong salting-in effect. This must result from the aromatic ring of phenol, because uncharged compounds without aromatic groups which increase the solubility of nonpolar compounds, such as ethanol, dioxane, and tetrahydrofuran, have almost no effect on the activity coefficient of ATGEE even at a concentration of 3 *M*.[13]

(4) The presence of chlorine- or iodine-containing substituents in a compound augments the salting-in effect. This is illustrated by the strong salting-in effect of trichloroacetate and diiodosalicylate, compared to the salting-out effect of acetate and trimethylacetate and the smaller effects of benzoate and phenol.

(5) Within the series of alkali halides, there is a tendency toward salting in with increasing size of the anion. However, there is no general correlation of ionic size with salting-in effect. Although SCN^-, ClO_4^-, and I^- are large ions and cause strong salting in, CH_3COO^-, $H_2PO_4^-$, HSO_3^-, and BrO_3^- cause strong salting out. Furthermore, trichloroacetate causes strong salting in, while trimethylacetate causes salting out. There is no simple relationship of cation size to salting out effectiveness.

(6) Salts of polyvalent anions all salt out strongly. The effect of Na_2HPO_4 could not be determined accurately because of interference by hydrolysis of ATGEE at alkaline pH. An estimate of the effect of this compound, obtained from an experiment carried out at pH 7.8 with approximate corrections for hydrolysis, indicated that salting out by HPO_4^{2-} is roughly the same as that by SO_4^{2-}.

Effects of Salts on Proteins. The effects of salts on proteins will be discussed in terms of the model described in the previous paper.[13] This model is based upon the assumption that there is a progressive increase in the exposure of portions of the protein to the solvent in the following series: solid protein < aggregated or polymeric protein < native monomeric protein < denatured protein. The effects of solvents on the physical state of the protein will depend upon the extent to which the solvent stabilizes, or decreases the activity coefficient of groups on the protein which become exposed to the solvent in a particular step. These effects may be divided into three main groups: (1) effects on charged groups, (2) effects on nonpolar, "hydrophobic" groups, and (3) effects on peptide and amide groups.

(*1*) *Effects on Charged Groups.* The charged groups of proteins are largely on the outside of the native protein,[18] so that they are exposed to the solvent in both the native and denatured state. Concentrated salt solutions, therefore, will interact in a similar manner with both native and denatured protein and would not be expected to have a large effect upon denaturation by an interaction with charged groups. The clearest evidence for this conclusion comes from the experiments of Bello, Riese, and Vinograd, who showed that the effects of salts on the melting of gelatin gels are not appreciably affected by removal of the charged groups on the protein, either by wide variation of the pH or by chemical blocking.[11] The salting out of hemoglobin is also not appreciably affected by

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variation of the pH over a somewhat narrower range,[19] and the effects of concentrated salt solutions on the depolymerization of F-actin cannot easily be accounted for solely by ion binding to charged groups.[6]

(2) *Nonpolar Groups.* Concentrated salt solutions will certainly affect protein denaturation by their stabilizing or destabilizing effect on nonpolar groups, which are largely buried in the interior of native, soluble proteins,[18b] but become more exposed to the solvent upon denaturation. However, the large amount of data which is available regarding the effects of salt solutions on the activity coefficients of nonpolar compounds[10] indicates that such effects do not account for the observed effects of concentrated salt solutions on the physical state of proteins. For example, the sodium and potassium salts of I^-, NO_3^-, and ClO_4^- and the chlorides of Ba^{2+}, Ca^{2+}, and Mg^{2+} increase the activity coefficients of benzene and other nonpolar or slightly polar compounds[20] and would, therefore, be expected to prevent denaturation by inhibiting the exposure of nonpolar groups of the protein to the solvent; in fact, these salts favor denaturation.[3-5] Activity coefficient effects on nonpolar compounds show a large sensitivity to the nature of the alkali cation, whereas salt effects on proteins show very little sensitivity to the nature of the alkali cation.[1,3,5,6,10] Finally, tetramethylammonium ion has a strong salting-in effect on nonpolar compounds but has very little effect on proteins.[5,6,10,21]

(3) *Peptide and Amide Groups.* The effect of concentrated salt solutions on the activity coefficient of ATGEE was examined in order to obtain evidence from a model compound for the effects of salts on the peptide and amide groups of a protein. With few exceptions, the effects of salts on the activity coefficient of ATGEE parallel closely their effects on protein denaturation, dissociation, and solubility. The effects of salts on the salting out and denaturation of proteins and on the activity coefficient of ATGEE are compared in Tables II–IV.

In Table II Hofmeister's data on the concentrations of salt required to cause precipitation of euglobulin[1] are compared to the salting out constants for ATGEE. The order of effectiveness is the same and in both groups the insensitivity to the nature of the alkali cation is evident. The salting-out constants for two proteins, hemoglobin and fibrinogen,[19,22] are compared to those for ATGEE in Table III. Although we do not believe that an exact quantitative comparison is justified at this time, it is of interest that the salting-out constants for hemoglobin are only about five times larger than those for ATGEE, and it might be suggested that the salting out of hemoglobin could be accounted for if the equivalent of approximately five ATGEE units were removed from exposure to the solvent upon precipitation of the protein. The K_s values for fibrinogen are approximately twice those for hemoglobin and the sensitivity to precipitation of this protein corresponds to that of approximately ten ATGEE units. Salts which cause an increase in the solubility of proteins and polypeptides in concentrated solution, such as LiBr and LiI, also increase the solubility of ATGEE and diketopiperazine.[12] The inhibition of certain antigen–antibody reactions by concentrated salt solutions has been ascribed to an interaction of ions with charged groups[23]; however, the order of anion effectiveness is $SCN^- > I^- > Br^- > Cl^- > F^-$, and this inhibition may be the result of a conformation change or some other activity coefficient effect caused by the salts.

The results of a few of the large number of studies of the effects of salts on protein denaturation[3-5,11,21,24,25] are shown in Table IV. Salts which salt out ATGEE tend to prevent denaturation and salts which salt in ATGEE favor denaturation. Polyvalent anions, acetate, fluoride, and chloride, which increase the activity coefficient of ATGEE, inhibit the denaturation of edestin, ovalbumin, collagen, and ribonuclease, while large halide anions and other anions which favor the denaturation of these proteins decrease the activity coefficient of ATGEE with an almost identical order of effectiveness. Ba^{2+}, Ca^{2+}, Mg^{2+}, and Li^+ favor denaturation and tend to salt in ATGEE, while the other alkali cations show little difference in their effects on proteins or on ATGEE. Very similar results have been obtained in other studies

Table II. Salting Out of Euglobulin

Salt	M^a	K_s of ATGEE
Na_3 citrate	0.56	+0.90
Li_2SO_4	0.78	
Na_2SO_4	0.80	0.48
K_2HPO_4	0.81	
Na_2HPO_4	0.82	~0.5
$(NH_4)_2SO_4$	1.01	0.45
$MgSO_4$	1.32	
KAc	1.67	
NaAc	1.68	0.23
NaCl	3.6	0.046
$NaNO_3$	5.4	−0.075

[a] Concentration of salt required to initiate precipitation; see ref. 1.

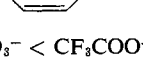
Table III. Salting-Out Constants, K_s, for Proteins and ATGEE[a]

Salt	Carboxy-hemo-globin[b]	Fibrino-gen[c]	ATGEE
NaCl		1.07	+0.046
$MgSO_4$	1.32		
$(NH_4)_2SO_4$	2.13	4.38	+0.45
Na_2SO_4	2.28		+0.48
Phosphate	3.00[d]	6.48[d]	+0.5[e]
			+0.36[f]
Na_3 citrate	4.14		+0.90

[a] Based on molar concentrations. [b] See ref. 19. [c] Data of M. Florkin, *J. Biol. Chem.*, **87**, 629 (1930), quoted in E. J. Cohn and J. T. Edsall, "Proteins, Amino Acids and Peptides," Reinhold Publishing Corp., New York, N. Y., 1943, p. 604. [d] Potassium phosphate, mole fraction K_2HPO_4 = 0.52. [e] Approximate value for Na_2HPO_4. [f] For NaH_2PO_4.

(19) A. A. Green, *J. Biol. Chem.*, **93**, 495 (1931).
(20) G. E. Boyd, S. Lindenbaum, and G. E. Myers, *J. Phys. Chem.*, **65**, 577 (1961); R. L. Bergen, Jr., and F. A. Long, *ibid.*, **60**, 1131 (1956); G. Akerlof, *J. Am. Chem. Soc.*, **57**, 1196 (1935).
(21) P. H. von Hippel and K.-Y. Wong, *Biochemistry*, 1, 664 (1962).
(22) See Table III, footnote *c*.
(23) W. J. Kleinschmidt and P. D. Boyer, *J. Immunol.*, **69**, 247, 257 (1952); D. Pressman, A. Nisonoff, and G. Radzimski, *ibid.*, **86**, 35 (1961).
(24) See Table IV, footnote *e*.
(25) See Table IV, footnote *f*.

Table IV

Inhibits denaturation ←	Increases denaturation →
Edestin, ovalbumin[a] $Fe(CN)_6^{4-} > citrate^{3-} > SO_4^{2-} > Ac^- > Cl^-$	$NO_3^- < Br^- < I^- <$ salicylate$^-$
Ovalbumin[b] $SO_4^{2-} > HPO_4^{2-}, Ac^-, Cl^- >$ citrate^{3-}	$NO_3^- < I^- < SCN^- <$ 
Collagen,[c] gelatin[c] SO_4^{2-}, AcO^-, F^-, $(CH_3)_4N^+$	Cl^-, $Me_3CCOO^- < Br^-$, $NO_3^- < CF_3COO^- < I^-$, SCN^-, $C_6H_5SO_3^-$, CCl_3COO^- < salicylate$^-$ < diiodosalicylate$^-$ $NH_4^+ \sim Rb^+ \sim K^+ \sim Na^+ \sim Cs^+ < Li^+ <$ $Mg^{2+} < Ca^{2+} < Ba^{2+}$
Ribonuclease[d] $\{HPO_4^{2-}, H_2PO_4^-\} > SO_4^{2-} > Cl^-$	$Br^- < ClO_4^- < SCN^-$ $(CH_3)_4N^+ \sim NH_4^+ \sim K^+ \sim Na^+ < Li^+$
DNA[e]	Cl^-, $Br^- < CH_3COO^- < I^- < ClO_4^- < SCN^- <$ CCl_3COO^- $(CH_3)_4N^+ \sim K^+ \sim Na^+ \sim Li^+$
Fumarase (activation)[f] Citrate$^{3-} > SO_4^{2-}$	(Inhibition) $Cl^- < Br^- < SCN^- < I^-$

[a] Ref. 3. [b] Ref. 4. [c] Ref. 11 and 21. [d] Ref. 5. [e] K. Hamaguchi and E. P. Geiduschek, *J. Am. Chem. Soc.*, **84**, 1329 (1962). [f] V. Massey, *Biochem. J.*, **53**, 67 (1953).

of protein denaturation.[26] As noted above, the effects of Mg^{2+}, Ca^{2+}, $(CH_3)_4N^+$, I^-, and ClO_4^- on proteins are in the opposite direction to their effects on most nonpolar organic solutes, but they are in the same direction as their effects on ATGEE.[3,5,10,11,21] Salt effects on proteins are more closely correlated with those on ATGEE than with those on benzoic acid,[5] which is strongly salted in by $(CH_3)_4N^+$ and displays a large sensitivity to the nature of different alkali cations. It is of interest that with ATGEE, as well as in the effect on the collagen–gelatin transition[21] and the depolymerization of actin,[6] there is a *decrease* in salting in (lowering T_m, favoring depolymerization) in going from ammonium to tetramethylammonium salts, followed by an *increase* in going from tetramethyl- to tetraethylammonium salts. The inhibition of a number of enzymes by relatively concentrated salt solutions, which may involve a conformational change to a less active form, shows a similar order of sensitivity to the nature of the salt.[26a,26b,27] Fumarase is inhibited by salts with the same order of effectiveness of anions as is observed toward the activity coefficient of ATGEE, and is activated by citrate and sulfate, which salt out ATGEE (Table IV).[25] The contraction of fibrous proteins, which occurs by a salt-induced phase transition from a crystalline to an amorphous state, shows a similar order of sensitivity for the limited number of salts which have been examined.[28] Polyproline exhibits an increase in solubility and a change of conformation in the presence of LiBr, $LiClO_4$, NaSCN, and $CaCl_2$, all of which are salts which might be expected to interact with the amide bonds of the polypeptide.[29] Surprisingly, even the order of denaturing effectiveness of concentrated salt solutions[24] toward DNA is similar to the order of effectiveness toward ATGEE. Hamaguchi and Geiduschek[24] rejected an explanation based upon salt effects on the activity coefficients of the bases for DNA denaturation because of the absence of a correlation with the order of salt effects on nonpolar organic molecules, which is most serious for $(CH_3)_4N^+$. However, nonpolar organic solutes may not be a satisfactory model for the bases of DNA, which contain polar groups with some resemblance to the amide groups of ATGEE, and the possibility that the salt effects on DNA result from salting in of the bases should be reconsidered.

The effect of concentrated salt solutions on the depolymerization of proteins is discussed in the following paper[6]; it may be noted here that the effectiveness of salts in depolymerizing F-actin to the monomer, G-actin, also parallels the effect of these salts on the activity coefficient of ATGEE.

The above facts and the difficulty of explaining salt effects on proteins by effects on charged or nonpolar groups lead to the conclusion that a major part of the effect of concentrated salt solutions on the denaturation, solubility, and dissociation of proteins may be accounted for by effects of salts on those peptide and amide groups of a protein which undergo a change in their degree of exposure to solvent when the protein undergoes a change in physical state.

Mechanism of the Effect of Salts on ATGEE and Proteins. The following mechanisms may be considered as possible explanations for the effects of salts on ATGEE; similar arguments may be made for effects on proteins, but the discussion will generally be restricted to the model peptide for simplicity.

(1) Removal of Solvating Water from the Peptide. Concentrated salt solutions do not exert their effects by changing the number of water molecules which are available to solvate amide groups, because there is no correlation of their effect on ATGEE with their effect

(26) (a) Y. Tonomura, K. Sekiya, and K. Imamura, *J. Biol. Chem.*, 237, 3110 (1962); (b) M. London, R. McHugh, and P. B. Hudson, *J. Gen. Physiol.*, **46**, 57 (1962); (c) K. Hamaguchi, A. Kurono, and S. Goto, *J. Biochem.* (Tokyo), **54**, 259 (1963).

(27) W. J. Rutter, *Acta Chem. Scand.*, 11, 1576 (1957); K. V. Rajagopalan, I. Fridovich, and P. Handler, *J. Biol. Chem.*, **236**, 1059 (1961); M. Coval, Ph.D. Thesis, Brandeis University, 1964.

(28) L. Mandelkern, W. T. Meyer, and A. F. Diorio, *J. Phys. Chem.*, **66**, 375 (1962); L. Mandelkern, J. C. Halpin, A. F. Diorio, and A. S. Posner, *J. Am. Chem. Soc.*, **84**, 1383 (1962).

(29) W. F. Harrington and M. Sela, *Biochim. Biophys. Acta*, 27, 24 (1958); I. Z. Steinberg, W. F. Harrington, A. Berger, M. Sela, and E. Katchalski, *J. Am. Chem. Soc.*, **82**, 5263 (1960).

on the activity of water. For example, the activities of water in 1 *m* solutions of NaCl, NaBr, and NaI are 0.9669, 0.9661, and 0.9649, respectively,[30] although NaCl causes salting out, NaI causes salting in, and NaBr has little effect on ATGEE. Furthermore, the solubility of the peptide shows little sensitivity to the nature of the alkali cation, although the different cations differ in their effects on the activity of water, and Li^+, Mg^{2+}, and Ca^{2+}, which markedly *decrease* the activity of water, *increase* the solubility of the peptide.

(*2*) *Classical Electrostatic Salting Out.* Electrostatic theories for salting out of the kind developed by Debye and Kirkwood do not account for the large differences between the effects of different salts on the salting out of nonpolar molecules and, in particular, do not account for changes from salting out to salting in as the nature of the salt is varied.[10] For the same reasons, and also because of the small effect of changes in the nature of the cation, such theories do not account for the effects of salts on ATGEE.

(*3*) *Water Structure.* Salts affect the "structure" of liquid water and, to the extent that such structural changes change the free energy of interaction with the solvent of groups on the protein which become exposed to solvent, they would be expected to influence the physical state of proteins by effects on water structure. Although solvent effects on proteins have frequently been attributed to changes in water structure, such suggestions have not always been accompanied by experimental support nor by a clear explanation of the reason for the postulated change in free energy of the protein–solvent interaction. Effects of salts on water structure do not provide a satisfactory explanation for the effects of salts on ATGEE (and for the effects on a number of proteins) at the present time for the following reasons.[31]

(*a*) Although there is some quantitative disagreement, most experimental measurements of the effects of salts on water structure that do not depend upon specific anion- or cation-induced effects show that water structure is dependent on the nature of both the anion and the cation. For example, two relatively straightforward measures of water structure, the viscosity *B* coefficient and the unitary partial molal entropy of solution of a salt, show differences in the series Na^+, K^+, and Cs^+ which are at least as large as those in the series Cl^-, Br^-, and I^- (Table V).[8] Measurements of the effects of salts on the self-diffusion of water and on proton spin-lattice relaxation times also show large differences in the effects of both alkali cations and halide anions.[32] If the effects of salts on ATGEE were mediated through effects on water structure, variation of the cation should have at least as much effect as variation in the anion. In fact, there is no difference in the effects of Na^+, K^+, and Cs^+ salts on ATGEE, whereas there are large differences between the effects of Cl^-, Br^-, and I^- salts.

Table V. Effects of Ions on "Water Structure"[a]

Ion	Viscosity *B* coefficient	Unitary partial molal entropy, e.u.
Li^+	0.15	−8.8
Na^+	0.09	+0.5
K^+	−0.01	10.7
Cs^+	−0.05	18.3
Cl^-	−0.01	11.0
Br^-	0	17.1
I^-	−0.08	22.8

[a] See ref. 8.

(*b*) In several instances the effects of particular ions are in the opposite direction from that required by the water structure theory. Most large anions are structure breaking and salt in ATGEE, but lithium ion, which is structure making,[8,33] also salts in ATGEE. Cesium ion is strongly structure breaking, while tetraalkylammonium ions are structure making,[7,8,34] yet neither has a marked effect on ATGEE. Furthermore, both acetate and trichloroacetate are "structure-making," as judged by the similar large increase in the viscosity of water observed in the presence of these ions,[35] but trichloroacetate decreases and acetate increases the activity coefficient of ATGEE.

(*c*) The enthalpy and entropy of interaction of sodium iodide with ATGEE are both negative (see below), whereas an effect mediated through a salt-induced disruption of water structure would be expected to exhibit a positive entropy and a small or positive enthalpy.

It is worthy of note that lithium bromide has a similar effect on the melting of elastoidin in water and in ethylene glycol solutions; the latter effects are clearly not mediated through water structure.[36]

(*4*) *Internal Pressure.* The effects of salts on the internal pressure or internal cohesion of water exhibit a satisfactory correlation with the differences in extent and direction of the effects of different salts on nonpolar solutes.[10,37] The internal pressure effect, or whatever similar effect is the correct explanation for salting out of organic solutes, certainly contributes to the effect of salts on ATGEE and is presumably the explanation for the salting *out* of this compound. The salting out constants, K_s, for ATGEE were calculated from eq. 2

$$K_s = \frac{V_i^0(V_s - \bar{V}_s^0)}{2.3RT\beta} \qquad (2)$$

in which V_i^0 is the molal volume of the solute, V_s is the molal volume of the "liquid" electrolyte, $\bar{V}^0$ is the partial molal volume of the electrolyte at infinite dilution, and β is the compressibility of water; the values were multiplied by 0.3 for reasons which are given elsewhere.[10,37] It is evident (Table VI) that the calculated K_s values[38] for salting out are much larger than the observed values and that the calculated values do not predict the salting in which is observed with

(30) C. C. Bigelow and I. I. Geschwind, *Compt. Rend. Trav. Lab. Carlsberg*, **32**, 89 (1961).

(31) A preliminary discussion of this point has been given: W. P. Jencks, *Federation Proc.*, **24**, Suppl. 15, 5 (1965).

(32) (a) J. H. Wang, *J. Phys. Chem.*, **58**, 686 (1954); (b) B. P. Fabricand, S. S. Goldberg, R. Liefer, and S. G. Ungar, *Mol. Phys.*, 7, 425 (1963).

(33) J. C. Hindman, *J. Chem. Phys.*, **36**, 1000 (1962), and references therein.

(34) E. R. Nightingale, Jr., *J. Phys. Chem.*, **66**, 894 (1962).

(35) B. Nagy, Ph.D. Thesis, Brandeis University, 1964.

(36) L. Mandelkern, G. Canty, and A. F. Diorio, *J. Phys. Chem.*, **67**, 2882 (1963).

(37) N. C. Deno and C. H. Spink, *ibid.*, **67**, 1347 (1963); F. A. Long and W. F. McDevit, *J. Am. Chem. Soc.*, **74**, 1773 (1952).

(38) See Table VI, footnote *a*.

salts of large anions, such as perchlorate, iodide, and benzoate. The discrepancy for salts which cause salting out may be accounted for by the polar nature of ATGEE, but the observed salting in by salts of certain large anions and by Li^+, Ca^{2+}, and Ba^{2+} is not accounted for by the internal pressure theory and requires another explanation.

Table VI. Observed and Calculated Salting-Out Constants, K_s, for ATGEE

Salt	ATGEE, K_s Calcd.[a]	ATGEE, K_s Obsd.
Na_2SO_4	2.6	0.48
$BaCl_2$	1.6	−0.11
NaCl	0.48	0.046
KCl	0.39	0.046
NaBr	0.51	0
LiCl	0.39	0.021
KBr	0.38	−0.023
$NaClO_4$	0.29	−0.33
NaI	0.31	−0.33
NH_4Cl	0.35	0.035
CsCl	0.34	0.054
C_6H_5COONa	−0.17	−0.14
$(CH_3)_4NBr$	−0.39	+0.018

[a] $K_s = 0.3V_i(V_s - \bar{V}_s^0)/2.3RT\beta$. A value of V_i of 286 ml./mole was estimated for ATGEE. This value was estimated from the relation $V = 0.42P$ (J. C. McGowan, *Rec. trav. chim.*, **75**, 193 (1956)) where P is the parachor. Parachor values were taken from O. R. Quayle, *Chem. Rev.*, **53**, 439 (1953). The value for the double-bond contribution of the amide group was estimated to be 15, from the reported values for acetamide and formamide.

(5) Direct Interaction of Salts with the Amide Groups of ATGEE. The failure of explanations based upon indirect effects of salts on the solvent suggests that salting in may be caused by direct interaction[12,39] of certain ions with ATGEE. An association of an ion with the peptide according to the equilibrium of eq. 3

$$P + X^- \rightleftharpoons PX^- \qquad K_1 = \frac{[PX^-]}{[P][X^-]} \qquad (3)$$

will result in an increase in peptide solubility which is linear with respect to salt concentration, with a slope equal to K_1. The effects of salts on activity coefficients which result from bulk solvent effects generally follow the Setschenow equation with a *logarithmic* relationship between solubility and salt concentration.[10] The majority of salts which cause salting in of ATGEE cause a linear increase in solubility with increasing salt concentration, from which values of K_1 were calculated (Table VII). Sodium trichloroacetate is an exception; the solubility increase is more nearly proportional to the square of the concentration of this salt, and it is possible that two molecules of trichloroacetate associate with the peptide. At 25° the total solubility remains linear with respect to salt concentration up to 3.3 M sodium iodide, and the calculated values of K_1 for the interaction of sodium iodide with ATGEE are 0.44, 0.56, and 0.97 M^{-1} at 40, 25, and 0°, respectively. Values of K_1 for other salts are given in Table VII. The free-energy changes which result from the binding of salt to a single site in 1 and 3 M salt solution, calculated from the equation[40]

(39) G. M. Waind, *J. Chem. Soc.*, 2879 (1954); S. Glasstone and A. Pound, *ibid.*, **127**, 2660 (1925).

Table VII. Apparent Equilibrium Constants and Free Energies for Salt–Peptide and Phenol–Peptide Interaction, Treated as a Direct Binding

Salt	K, M^{-1}	$-\Delta F$,[a] cal./mole— 1 M	3 M
$CaCl_2$	0.22	120	300
LiBr	0.41	200	480
NaI, KI	0.56	260	580
KSCN	0.60	280	610
NaSCN	0.72	320	680
$NaClO_4$	0.75	330	700
LiI	0.82	350	740
$C_6H_5CH_2NH_3Cl$	0.88	370	760
Sodium tosylate	0.94	390	790
Phenol	1.4	520	980
Lithium diiodosalicylate	6.3	1170	1770

[a] $\Delta F = RT \ln (1 + K[\text{salt}])$,[40] calculated for 1 and 3 M salt.

$\Delta F = -RT \ln (1 + K[\text{salt}])$, are also given in Table VII. These numerical values should not be taken too seriously, but they do provide an indication of the magnitude of the changes in free energy of a peptide "site" to be expected in the presence of different salts. The effects of several of the salts on ethyl acetate have been examined[41] and are much smaller and sometimes in the opposite direction, compared to those on ATGEE, which indicates that most of the effect observed with ATGEE may be attributed to interaction with the amide portion of the molecule. The temperature dependence of the apparent equilibrium constant for complex formation with sodium iodide gives ΔH and ΔS values of approximately −3300 cal./mole and −9.9 e.u., respectively.

Experimental support for the hypothesis that there is a direct interaction between the anions and the dipoles of the amide groups of ATGEE comes from the fact that the order of effectiveness of ions in salting in ATGEE is similar to the order of anion binding in other systems. The binding of anions to anion exchange resins,[42] in which electrostatic interactions may be presumed to play a major role,[43] follows a closely similar order to that for interaction with ATGEE (Table VIII). In both cases the anion may interact with more than a single positive site. The fact that sulfate does not salt in ATGEE is not inconsistent with this correlation, because the tight binding of sulfate to ion-exchange resins is largely a mass law effect resulting from its two charges, and at concentrations above 0.05 to 0.18 M sulfate is bound less tightly than chloride to ion exchangers.[44] The order of interaction of anions with ATGEE is essentially the same as the order of binding of anions to cationic sites on proteins: CCl_3COO^- > tosylate$^-$ > SCN^- > ClO_4^- > I^- > NO_3^- > Br^- > Cl^- > CH_3COO^-.[45] A similar order, with some exceptions which may reflect specific steric re-

(40) J. A. Schellman, *Compt. Rend. Trav. Lab. Carlsberg*, **29**, 230 (1955).
(41) A. P. Altshuller and H. E. Everson, *J. Am. Chem. Soc.*, **75**, 4823 (1953).
(42) See Table VIII, footnote *a*.
(43) S. A. Rice and F. E. Harris, *Z. Physik. Chem.* (Frankfurt), **8**, 207 (1956).
(44) R. M. Wheaton and W. C. Bauman, *Ind. Eng. Chem.*, **43**, 1088 (1951); R. Kunin, "Ion Exchange Resins," 2nd Ed., John Wiley and Sons, Inc., New York, N. Y., 1958. p. 61.
(45) J. Steinhardt, C. H. Fugitt, and M. Harris, *J. Res. Natl. Bur. Std.*, **28**, 201 (1942); G. Scatchard and E. S. Black, *J. Phys. Chem.*, **53**, 88 (1949); L. G. Longsworth and C. F. Jacobsen, *ibid.*, **53**, 126 (1949).

Table VIII. Affinity of Anions for Anion-Exchange Resins

Ion	K^a	ATGEE, K_s for Na salt
ClO_4^-	32	−0.33
SCN^-	19	−0.25
$(CH_3)_3CCOO^-$	18	−0.27
p-Tos^-	14	−0.31
I^-	8–13	−0.23
Br^-	3.4	0
NO_3^-	3.3	−0.075
Cl^-	1.0	0.046
BrO_3^-	1.0	+0.090
$H_2PO_4^-$	0.34	+0.36
CH_3COO^-	0.17	+0.23
F^-	0.10	+0.23[b]

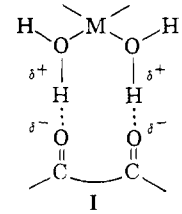
[a] Relative affinity for the quaternary ammonium resin, Dowex-2: S. Peterson, *Ann. N. Y. Acad. Sci.*, **57**, 144 (1954); H. P. Gregor, J. Belle, and R. A. Marcus, *J. Am. Chem. Soc.*, **77**, 2713 (1955). [b] Potassium salt.

quirements, is found for the inhibition by relatively dilute salt solutions of a number of enzymes, which presumably involves binding to a charged group at the active site.[46] Furthermore, there is direct evidence for the binding of triiodide, thiocyanate, and aromatic anions to the uncharged, amide-containing polymer, polyvinylpyrrolidone; this binding has been attributed to ion–dipole and van der Waals' forces.[47]

There has been a controversy over the question of whether the effects of salts on gelatin result from a binding of ions to peptide groups.[11,21] It has recently been pointed out that the observed effects could be accounted for by assuming reasonable values for binding constants for such an interaction[48]; these binding constants are similar to or somewhat smaller than those suggested for ATGEE (Table VII).

The high activity of large halide and halide-containing anions in salting in ATGEE suggests the existence of a van der Waals–London type of interaction with the peptide, which is dependent on the polarizability of the anion. However, the fact that a correlation with at least the gross polarizability of the ion does not hold in all cases (*e.g.*, acetate and trimethylacetate) suggests that a direct electrostatic interaction with the large dipoles of the amide groups[49] of ATGEE is also involved. It may appear surprising, at first sight, that an electrostatic interaction should be greater for large than for small anions, especially in view of the fact that it is generally believed that most anions are not surrounded by a firmly bound sheath of water of hydration.[33,50] However, the interaction of a large anion with ATGEE will be accompanied by a decrease in the interaction of the ion with water and, as in the binding of ions to ion-exchange resins and in ion pair formation,[51] the energy of ion binding to an amide dipole represents a small difference between the much larger energies of ion hydration and ion–dipole interaction. It is not unreasonable that this balance of energies should favor the binding of large anions to an amide dipole in view of (a) the experimental evidence cited above for the order of the binding of anions to positively charged sites, (b) the sensitivity of the energy of interaction of ions with the dipole of water to ionic radius, which has been reported to include quadrupole interaction terms and to be approximately proportional to $(r + a)^{-3}$, in which r is the ionic radius and a is the effective radius of the water molecule, 1.38 Å.,[52] and (c) the fact that a close interaction of an ion with a large dipole may be considered in terms of the separate forces exerted by the two charges of the dipole. The interaction with an amide dipole is somewhat analogous to the interaction of an ion with the surrounding charges in a crystal lattice, and it is known that the close balance between crystal lattice and hydration energies favors the binding of large anions to the crystal lattice.[53] Thus, the postulated direct interaction of large anions with ATGEE is more a reflection of the relatively weak interaction of these ions with water than of a strong direct interaction with the peptide. In the series of oxyanions, an increase in the charge (as in SO_4^{2-}), a reduction in the number of oxygen atoms over which the charge is distributed (compare ClO_4^-, BrO_3^-, and CH_3COO^-), an increase in basicity, or the addition of hydroxyl groups (compare ClO_4^- and $H_2PO_4^-$) would be expected to increase the strength of the interaction with water and make it correspondingly more difficult for the ion to interact with the peptide.

The tendency toward salting in of ATGEE by salts of lithium and divalent cations may also be interpreted in terms of a direct interaction, which would be expected to involve the cation hydrate for these ions. The structure of such a hydrate is similar to that of compounds of the urea–guanidinium class and polyfunctional interaction may occur in the same manner as postulated[13] for these compounds (I). There is abundant evidence for direct interaction of lithium ion



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with amide or amide-like groups in the absence of water[54] but, with the exception of nuclear magnetic resonance evidence that such an interaction persists in concentrated aqueous dimethylformamide,[55] the

(46) (a) E. Walaas and O. Walaas, *Acta Chem. Scand.*, 10, 122 (1956); (b) I. Fridovich, *J. Biol. Chem.*, **238**, 592 (1963).

(47) (a) W. von Scholtan, *Makromol. Chem.*, **11**, 131 (1953); (b) H. P. Frank, S. Barkin, and F. R. Eirich, *J. Phys. Chem.*, **61**, 1375 (1957); (c) P. Molyneux and H. P. Frank, *J. Am. Chem. Soc.*, **83**, 3169, 3175 (1961).

(48) J. Bello, *Biochemistry*, **2**, 276 (1963); L. Mandelkern and W. E. Stewart, *ibid.*, **3**, 1135 (1964).

(49) L. Pauling in "Symposium on Protein Structure" A. Neuberger, Ed., John Wiley and Sons, Inc., New York, N. Y., 1958; P. Haake, W. B. Miller, and D. A. Tyssee, *J. Am. Chem. Soc.*, **86**, 3577 (1964).

(50) J. E. Desnoyers and B. E. Conway, *J. Phys. Chem.*, **68**, 2305 (1964); but see J. Padova, *J. Chem. Phys.*, **40**, 691 (1964).

(51) B. Chu, D. C. Whitney, and R. M. Diamond, *J. Inorg. Nucl. Chem.*, **24**, 1405 (1962); D. R. Rosseinsky, *J. Chem. Soc.*, 785 (1962).

(52) A. D. Buckingham, *Discussions Faraday Soc.*, **24**, 151 (1957); H. F. Halliwell and S. C. Nyburg, *Trans. Faraday Soc.*, **59**, 1126 (1963).

(53) H. F. Halliwell and S. C. Nyburg, *J. Chem. Soc.*, 4603 (1960).

(54) I. I. Geschwind, *Nature*, **187**, 324 (1960); J. Bello and H. R. Bello, *ibid.*, **190**, 440 (1961); J. Bello and H. R. Bello, *ibid.*, **194**, 681 (1962); G. Sarda and N. Peacock, *ibid.*, **200**, 67 (1963); W. F. Harrington, Abstracts, 147th National Meeting of the American Chemical Society, Philadelphia, Pa., April 1964, p. 10H; J. M. Bryant and W. W. Wendlandt, *Science*, **123**, 897 (1956), reported an effect of very dilute lithium chloride on the viscosity and refractive index of urea solutions, which was interpreted as evidence for complex formation; we have attempted to repeat the viscosity measurements reported by these workers, but have been unable to make measurements of sufficient accuracy to reproduce the small effects reported.

(55) R. A. Craig and R. E. Richards, *Trans. Faraday Soc.*, **59**, 1972 (1963).

existence of a lithium ion effect on amide groups in the presence of water has not been clearly demonstrated previously.

An alternative explanation of the interaction of ions with ATGEE may be based upon an examination of the properties of the surface layer of water which surrounds the solute, as Sinanoğlu and Abdulnur have done for the bases of DNA.[56] Cations are almost completely excluded from an air–water or hydrocarbon–water interface because of their requirement for hydration, whereas the less hydrated anions exhibit a large variation in the degree of their exclusion, and may even be concentrated on the surface.[57] Relocation of these excluded ions upon solution of a nonpolar solute, which is primarily an entropy effect, requires work and will contribute to the salting out of such solutes (Figure 4A). In the case of a polar solute, such as ATGEE, ions will be less excluded from the surface and weakly hydrated anions may even be concentrated at the surface to cause salting in (Figure 4B). The cations Na^+, K^+, Rb^+, Cs^+, and NH_4^+ are all excluded equally and almost completely from the air–water and air–hexane interface.[57] The order of exclusion of anions from the air–water interface is $F^- > OH^- > Cl^- > BrO_3^- > Br^- > NO_3^- > I^- > ClO_4^- > SCN^-$,[57] which agrees closely with the order of increasing salting-in effectiveness toward ATGEE. This approach, therefore, accounts for the insensitivity of the salting out of ATGEE to the nature of the alkali cation and the large differences in effectiveness, as well as the order of effectiveness, of anions toward this peptide.

Interaction of the Peptide Group with Aromatic and Polarizable Groups. The strong salting-in effects of ions containing aromatic groups and of phenol toward ATGEE is evidence for an interaction, in aqueous solution, of aromatic groups with the peptide group, since no such salting in is seen with ions containing large aliphatic groups nor with aliphatic solvents. The apparent equilibrium constants and the free energies of interaction of 1 and 3 *M* solutions for phenol and some aromatic anions with ATGEE are given in Table VII. The existence of such an interaction is of interest because of its possible importance in the maintenance of native protein structure and in the interaction of proteins with solvents. It provides a partial explanation, for example, for the well known denaturing and solubilizing action of phenol toward proteins,[58] the remarkable effectiveness of *p*-nitrophenolate ion in potentiating the denaturation of ovalbumin,[4] and the fact that aromatic anions are especially effective in lowering the melting point of gelatin gels.[11] The fact that a number of proteins enter the phenol phase upon partitioning between phenol and water is evidence that the activity coefficients of a large number of the constituent parts of the protein must be lower in phenol than in water and may result, in part, from a phenol–amide interaction.[59]

Some experimental indications of the existence of an interaction between amide and aromatic groups have been reported previously. Refractive index measurements on solutions of simple amides and such aromatic compounds as phenol, in water and other solvents, have suggested the existence of a 1:1 complex of the two solutes.[60] The nuclear magnetic resonance and infrared spectra of N-methylamides in nonaqueous solvents show changes in the presence of aromatic compounds which have been interpreted as evidence for complex formation.[61] Neutral as well as anionic aromatic molecules bind to polyvinylpyrrolidone, and it has been suggested that the thermodynamic parameters of this binding indicate an exothermic interaction of the π-electron systems of the aromatic and amide groups, in addition to hydrophobic binding in this system.[47c] Nylon and wool show a strong adsorption of phenol from aqueous solution and an adsorption of benzene from butanol solution, which suggests the existence of an interaction between the aromatic ring and the amide groups of the polymer, although hydrogen bonding may also be involved in the binding of phenol.[62]

The aromatic–amide interaction may be ascribed to an interaction of the polarizable π-electrons of the aromatic ring with the amide[63] or, possibly, to the formation of a weak molecular complex between the amide and unoccupied orbitals of the aromatic system.

Acknowledgments. We are indebted to colleagues, too numerous to enumerate, for many useful discussions of the material in this and the preceding paper. We are grateful to the National Institutes of Health (Grants No. HD-01247 and AM-04501), the National Science Foundation (Grant No. GB-1648), and the National Institutes of Health, Division of General Medical Sciences (Training Grant No. 5 T1-GM-212-04) for generous financial support.

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