The Effects of Salts on the Free Energy of the Peptide Group[1,2]

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Abstract: The effects of several salts on the activity coefficients of the *N*-acetyl ethyl esters of glycine, diglycine, and triglycine, and on the simple amides, formamide, acetamide, and *N*-methylacetamide, have been determined at 0.5, 25.0, and 40.0° from distribution and solubility measurements, at salt concentrations up to 2.0 *M*. The three glycine derivatives and acetyltetraglycine ethyl ester are a series of compounds differing in the number of peptide groups (CH_2CONH) which they contain. Therefore the differences between the salt effects on these four compounds represent the effects on the peptide group, and they should apply to the repeating peptide unit in proteins. The peptide group is strongly salted *in*, or stabilized, by NaI, $NaClO_4$, NaSCN, $NaCl_3CCOO$; it is salted in less strongly by KF, LiCl, NaCl, KCl, CsCl, and NaBr; and $(CH_3)_4NBr$ and Na_2SO_4 have negligible or weak salting in effects. Free energies of transfer of the peptide group from water into 2.0 *M* salt solutions at 25° for the first two groups of salts are −170 to −215 and −55 to −100 cal/mol, respectively. The salt effects on the peptide group are generally additive in these compounds suggesting that these effects may be additive in proteins. The effects of salts on formamide are similar to effects on the peptide group with most salts. The effects of temperature are small but there is a trend toward increasing salting in with decreasing temperature for the peptide group and formamide. The results indicate that any change in protein structure resulting in an increased exposure of peptide groups to the solvent (*e.g.*, denaturation) will be favored by most salts through salt–peptide group interactions, and these interactions can provide large driving forces. The peptide group effects will usually be opposed by salting out effects on amino acid side chains, especially nonpolar groups.

Concentrated salt solutions have large effects on the structure and properties of proteins, including their solubility, denaturation, dissociation into subunits, and the activity of enzymes.[4] These effects are sensitive to the nature of the salt and may vary over a wide range, even for salts of the same charge type. The order of effectiveness of different salts on proteins is generally similar to the Hofmeister series which was described for the salting out of proteins nearly a century ago,[5] but in spite of much investigation the mechanisms of these effects remain poorly understood.

Because of the complexity of proteins, a variety of different interactions with salts may occur and it is difficult to determine which interactions are important for the reactions of proteins. One approach to the investigation of these problems is to determine the effects of salts on the free energies of model compounds which contain components of proteins such as peptide or nonpolar groups. In this way it may be possible to sort out the contributions of different components of proteins to their salt effects. It is a basic assumption in this approach that the effects of salts on components of model compounds are similar to their effects on those components in proteins. This requires that various group effects be additive, or at least that any lack of additivity in proteins be also observed in model compounds. It may be difficult to prove that group effects in proteins are additive, but support for the assumption of additivity may be obtained if it can be shown that certain group effects are additive in model compounds.

Previous investigations of model compounds have provided indications of the contributions of peptide and amide groups to salt effects on proteins. The activity coefficients of acetyltetraglycine ethyl ester (AG_4E), a model for peptide groups in proteins, have been measured in a large number of salt solutions and reveal a good correlation with salt effects on proteins.[6] Salts with the strongest salting *in* effects on AG_4E generally have the strongest tendencies to denature, dissociate into subunits, and solubilize proteins, while salts which salt *out* AG_4E have the opposite effects. It was concluded that interactions of salts with peptide groups largely account for the former effects on proteins but it was not possible to completely separate the contributions of peptide groups from those of other components of AG_4E. Subsequently, Schrier and Schrier reported salt effects on the activity coefficients of *N*-methylacetamide and *N*-methylpropionamide.[7] Assuming that the hydrocarbon and amide group effects are additive, these authors calculated amide group contributions for several salts and also concluded that denaturing effects of inorganic salts can be attributed to their salt–peptide interactions. They pointed out, however, that further studies on model compounds were needed, particularly with respect to the assumption of additivity of different group effects.

We report here an investigation of the effects of several salts on the activity coefficients of a series of com-

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(2) For the purposes of this report the *peptide group* is defined by the formula, CH_2CONH or $NHCH_2CO$, which represents the difference in stoichiometry between the successive members of the series of four peptide esters considered here, I. The peptide group, as defined here, contains one more hydrogen atom than the repeating peptide unit in proteins. The *amide group* refers to the amide (CONH) portion of the peptide group.

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(4) (a) P. H. von Hippel and T. Schleich in "Biological Macromolecules," Vol. II, S. Timasheff and G. Fasman, Ed., Marcel Dekker, New York, N. Y., 1969, p 417; (b) W. P. Jencks, "Catalysis in Chemistry and Enzymology," McGraw-Hill, New York, N. Y., 1969, p 351; (c) P. H. von Hippel and T. Schleich, *Accounts Chem. Res.*, 2, 257 (1969).

(5) F. Hofmeister, *Arch. Expt. Pathol. Pharmakol.*, 24, 247 (1888).

(6) D. R. Robinson and W. P. Jencks, *J. Amer. Chem. Soc.*, 87, 2470 (1965).

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

pounds which are models for the peptide and amide groups of proteins. In the accompanying paper, salt effects are reported on another series of compounds which are models for the hydrocarbon side chains in proteins.[8] The principle objectives of this work are as follows: (1) to determine the contribution of the peptide group to the free energies of model compounds in salt solutions in order to provide reasonable estimates for salt effects on peptide groups in proteins; (2) to determine whether the peptide group effects are additive in a series of model compounds in order to provide some basis for the assumption that peptide group effects in proteins are additive; (3) to attempt to separate salt effects on the amide portion of the peptide group from effects on neighboring nonpolar portions of peptides and amides; (4) to determine salt effects on *N*-acetylamino acid ethyl esters which are model compounds for nonpolar groups in proteins. The latter should provide information about the additivity of nonpolar and peptide group effects in model compounds, and presumably also in proteins.

Experimental Section

[^{14}C]Acetylglycine ethyl ester (AG_1E)[9] was prepared by the method of Wolf and Nieman.[10] Glycine ethyl ester hydrochloride (0.25 mol) was added to NaOH (0.20 mol) in 100 ml of water. With constant stirring, [1-^{14}C]acetic anhydride (0.25 mol) and NaOH (0.20 mol) were slowly added at -8 to $-10°$. The solution was neutralized to pH 7.0 by adding solid Na_2CO_3 and the product was extracted with ethyl acetate. The volume of ethyl acetate was reduced *in vacuo* until the product crystallized. The compound was recrystallized from ether, mp 46–47° (lit. 47–48.5°), specific activity 7.3 mCi/mol. The infrared spectrum ($CHCl_3$) shows strong bands at 3440, 3000, 1745, 1680, 1515, 1415, 1255, 1223, and 1040 cm^{-1}.

[^{14}C]Acetylglycylglycine ethyl ester (AG_2E) was prepared by slowly adding [1-^{14}C]acetic anhydride (0.17 mol) to a solution of glycylglycine ethyl ester hydrochloride (0.044 mol) in 100 ml of 40% aqueous pyridine at 0°, with constant stirring. The volume of the reaction mixture was reduced *in vacuo* and the product crystallized. It was recrystallized from ethanol, mp 146–148°, specific activity 2.6 mCi/mol. The infrared spectrum ($CHCl_3$) shows strong bands at 3433, 1748, 1675, and 1411 cm^{-1} and medium bands at 3323 (sh), 1538, 1494, 1298 (broad), and 1028 cm^{-1}.

Anal. Calcd for $C_8H_{14}N_2O_4$: C, 47.52; H, 6.98; N, 13.85. Found: C, 47.60; H, 7.05; N, 13.85.

[^{14}C]Acetylglycylglycylglycine ethyl ester (AG_3E) was prepared in the same manner as AG_2E. The product was recrystallized from water, mp 231–232°, specific activity 0.70 mCi/mol. The infrared spectrum (KBr) shows strong bands at 3296, 3074, 1742, 1670 (sh), 1634, 1554, 1402, 1229, and 1042 cm^{-1}.

Anal. Calcd for $C_{10}H_{17}N_3O_5$: C, 46.32; H, 6.62; N, 16.21. Found: C, 46.26; H, 6.64; N, 16.31.

[^{14}C]*N*-Methylacetamide was obtained from the reaction of 40% aqueous methylamine with [1-^{14}C]acetyl chloride at -10 to $-15°$.[11] The product was distilled from the reaction mixture, bp 110–112° (50 mm), specific activity 0.98 mCi/mol. The infrared spectrum was essentially identical with that of a pure sample of *N*-methylacetamide.

[^{14}C]Formamide was prepared from the reaction of ethyl formate (carboxyl ^{14}C) with concentrated aqueous ammonia at -8 to $-10°$.[12] The reaction mixture was distilled and a fraction collected, bp 106–110° (20 mm). This fraction was redistilled, bp 108–110° (20 mm) (lit. 111° (20 mm)).[13] The identity of the product was confirmed by the infrared spectrum.

[1-^{14}C]Acetamide was obtained commercially and recrystallized giving a preparation of specific activity 0.022 mCi/mol.

To demonstrate isotopic purity of these compounds, formamide and *N*-methylacetamide were redistilled and all of the other compounds were recrystallized with no significant change in specific activities in any case.

Reference phase solvents, chloroform and diethyl ether, were reagent grade materials used without further purification. The hexanol was Eastman practical grade in formamide experiments. Experiments of formamide with NaCl and KCl solutions were repeated using redistilled *n*-hexanol with identical results. Inorganic salts were analyzed reagents, except sodium perchlorate which was Fisher purified grade, and were used without further purification. Sodium trichloroacetate was prepared by neutralization of the reagent grade acid with sodium hydroxide. Tetramethylammonium bromide was recrystallized. Glass distilled water was used throughout. All melting points are uncorrected.

Radioactivity measurements were made with a Packard Model 3375 liquid scintillation spectrophotometer at 4°. Samples (0.10 ml) were added to vials containing 10 ml of scintillating solution of the following composition: naphthalene, 50 g; 2,5-diphenyloxazole, 7 g; *p*-bis[2-(5-phenyloxazolyl]benzene, 0.5 g; 95% ethanol, 133 ml; *p*-dioxane, 1.0 l. The samples were counted for sufficient time to accumulate >20,000 counts in most cases and >10,000 counts in all cases, reducing the standard errors of counting to <0.5% or <1%, respectively.

Infrared spectra were obtained with a Perkin-Elmer Model 221 infrared spectrophotometer.

Methods. In both distribution and solubility measurements, the compounds were equilibrated with solvents in 12-ml capacity tubes sealed with Teflon-lined screw caps. The tubes were submerged in a water bath in a rotating rack, and mixing was accomplished by rotating the tubes end-over-end at 25–30 rpm. Temperatures were maintained at 0.5 ± 0.05, 25.0 ± 0.05, and 40.0 ± 0.1°.

Distribution Experiments. The duration of equilibration was varied in occasional experiments in order to determine the time required for equilibrium to be reached, including the following systems: all compounds at 25° with water as the aqueous phase; AG_1E and AG_2E with water at 0.5 and 40° and 2.0 *M* NaCl at 0.5°; acetamide, NMA, AG_1E, and AG_2E with 1.0 *M* NaCl and 1.0 *M* Na_2SO_4 at 25°. These experiments demonstrated that equilibrium was reached within 20 min of equilibration at 25 and 40°, and within 1 hr at 0.5°. In all other experiments equilibrations were carried out for 1 hr at 25 and 40° and for 4 hr at 0.5°.

Following equilibration, the tubes were allowed to stand for 2 hr to allow separation of the phases before removing samples of each phase for assay. As a test that this period of time was sufficient to allow complete separation of the phases, several tubes containing different compounds and water as the aqueous phase were allowed to stand for periods up to 5 hr at 25 and 40°, and overnight at 0.5°, and the same results were obtained.

Solutions were assayed by removing 0.10-ml samples with micropipets from both phases in every case. Samples were removed from the denser phase in each tube by carefully introducing the tip of a pipet through the less dense phase into the underlying phase. It appears that any contamination of one phase with another during removal of samples for assay was not serious enough to affect the results significantly. The reproducibility of the distribution coefficients was reasonably good, as can be seen from the standard errors in Table I. Significant contamination of the phases would be expected to introduce more variability into the results.

The degree of mutual solution of the phases in these distribution experiments has not been directly measured. The solubility of several salts in water-saturated chloroform has been reported to be <0.005 *M*.[7] We have found that the solubilities of Na_2SO_4 and NaCl in water-saturated diethyl ether and *n*-hexanol solutions are <0.01 *M*, based on observation of excess solid phases after equilibration of weighed quantities of the salts with these solvents at room temperature (21 ± 1°). Mutual solution of the phases might be expected to be a significant problem with ether but not with hexanol. Water saturated with ether contains 6.03% ether (w/w) at 25°, but water saturated with *n*-hexanol contains only 0.63% hexanol.[14]

(8) P. K. Nandi and D. R. Robinson, *J. Amer. Chem. Soc.*, 94, 1308 (1972).

(9) Abbreviations are: AG_1E, acetylglycine ethyl ester; AG_2E, acetyldiglycine ethyl ester; AG_3E, acetyltriglycine ethyl ester; AG_4E, acetyltetraglycine ethyl ester.

(10) J. P. Wolf, III and C. Niemann, *Biochemistry*, 2, 493 (1963).

(11) G. F. D'Alelio and E. E. Reid, *J. Amer. Chem. Soc.*, 59, 109 (1937).

(12) L. F. Fieser and M. Fieser, "Organic Chemistry," 3rd ed, Reinhold, New York, N. Y., 1956, pp 178–179.

(13) J. R. A. Pollock and R. Stevens, Ed., "Dictionary of Organic Compounds," 4th ed, Vol. 3, Oxford University Press, New York, N. Y., 1965, p 1456.

(14) G. M. Bennett and W. G. Philip, *J. Chem. Soc.*, 1930 (1928); J. A. V. Butler, D. W. Thompson, and W. H. Maclennan, *ibid.*, 674 (1933).

Table I. Distribution Coefficients and Solubilities in Water[a]

Compd	Ref phase	0.5°	25.0°	40.0°
Acetylglycine ethyl ester	Chloroform	1.143 ± 0.024 (7)	0.814 ± 0.009 (15)	0.716 ± 0.006 (7)
Acetyldiglycine ethyl ester	Chloroform	34.53 ± 0.44 (7)	19.15 ± 0.38 (19)	14.44 ± 0.22 (8)
Acetyltriglycine ethyl ester	Solid phase	2.57 ± 0.14 (5)	6.48 ± 0.06 (8)	11.08 ± 0.45 (5)
Formamide	*n*-Hexanol		12.50 ± 0.21 (10)	12.18 ± 0.30 (8)
	Ether		129.1 ± 1.6 (7)	
Acetamide	Chloroform		80.84 ± 2.36 (8)	74.93 ± 1.95 (7)
N-Methylacetamide	Chloroform		13.37 ± 0.30 (8)	10.79 ± 0.15 (9)

[a] Distribution coefficients are C_{H_2O}/C_{ref}, the ratio of the concentrations of the nonelectrolyte in water and in the reference phase. Values for AG_3E are solubilities in grams per liter. Each value is a mean followed by the standard deviation of the determinations, and the numbers of determinations are given in parentheses.

Solubility Measurements. Solutions were equilibrated with an excess of AG_3E for periods of 10 days at 0.5°, 72 hr at 25°, and 24 hr at 40°. It was established that equilibrium had been reached within these time periods by two methods. In the first, duplicate tubes containing water and 2.0 *M* NaCl were equilibrated for further periods of 24–28 hr at 25 and 40° and 2–3 days at 0.5°, and the solubility was found to be the same as in samples equilibrated for the normal periods of time. In the second method, tubes containing water, 2.0 *M* NaCl, 2.0 *M* $NaClO_4$, and 1.0 *M* Na_2SO_4 were supersaturated by warming prior to equilibration. The concentrations of AG_3E in these tubes were found to be the same as in samples where equilibrium was approached from undersaturation. Following equilibration, rapid filtration of the samples was performed. Previous work has shown that rapid filtration of solutions of AG_4E did not introduce errors in the solubility determinations of that compound at 25°.[6] Filtration of solutions equilibrated at 0.5° was performed in a cold room at *ca.* 5°. A few tubes which had been equilibrated at 40° were allowed to stand until the solid phase appeared to have completely settled out, leaving clear solutions. Samples were removed and assayed from those tubes without prior filtration and gave results in good agreement with filtered samples.

Results[15]

The activity coefficients of AG_3E were determined from solubility measurements using eq 1, where f_i^0 and

$$f_i^0 C_i^0 = f_i^s C_i^s \quad (1)$$

f_i^s are activity coefficients in water and salt solution, respectively, and C_i^0 and C_i^s are the corresponding concentrations in moles per liter. The value of f_i^0 is assumed to be equal to 1.0, and therefore, $f_i^s = C_i^0/C_i^s$.[16]

Activity coefficients of all compounds except AG_3E were determined by distribution experiments. The distribution of a compound *i* between water and a reference phase is given by eq 2, where f_i^r is the activity co-

$$f_i^0 C_i^0 = f_i^r C_i^{r,0} \quad (2)$$

efficient in the reference phase and $C_i^{r,0}$ is the concentration in the reference phase in a system where the aqueous phase is water. The distribution of the compound between the same reference phase and a given salt solution is similarly described by eq 3. Combining eq 2 and 3, again assuming that f_i^0 is equal to 1.0, gives

$$f_i^s C_i^s = f_i^r C_i^{r,s} \quad (3)$$

eq 4, which relates the activity coefficient in salt solutions

$$f_i^s = (C_i^0/C_i^{r,0})/(C_i^s/C_i^{r,s}) \quad (4)$$

to the ratio of the two distribution coefficients. This relationship holds for varying concentrations of *i* only if the activity coefficient in the reference phase (f_i^r) is constant.[16] Distribution coefficients were measured between water and reference phases for all of the compounds over approximately tenfold ranges of concentrations which exceeded the ranges of concentration used in these experiments. In every case the distribution coefficients were found to be independent of concentration, demonstrating that the activity coefficients of all of the compounds in their reference phases were constant under the conditions of these experiments.

Distribution coefficients between water and reference phases and solubilities in water are reported in Table I. The range of error in each case was generally within ±5% of the reported mean values and it is assumed that the data in salt solutions have the same accuracy. In most cases each activity coefficient is based on a single determination in salt solution. Although each activity coefficient value incorporates the errors of two distribution or solubility measurements, the values are considered to have ranges of error generally within ±5% since distribution and solubilities in water are based on multiple determinations. In almost every case, activity coefficients were determined at at least four and usually five different salt concentrations over the range 0–2 *M*.

The effects of salt solutions on the activity coefficients of nonelectrolytes usually follow the Setchenow equation (eq 5), where C_s is the molar salt concentration,

$$\log f_i = k_s C_s \quad (5)$$

and k_s is the salting out constant which is characteristic for each salt, and provide a convenient basis for comparisons.[16,17] The present data frequently follow eq 5 over the entire range of salt concentration examined and, in these cases, salting out constants were determined by regression analysis with the aid of a computer. In the case of nonlinear plots, k_s values are based on initial slopes which were obtained graphically by passing the best straight line visually through points at salt concentrations up to and including 0.50 or 1.0 *M*. The salting out constants at 25° are given in Table II,

(15) Listings of activity coefficients will appear following these pages in the microfilm edition of this volume of the Journal. Single copies may be obtained from the Reprint Department, ACS Publications, 1155 Sixteenth St., N.W., Washington D. C. 20036, by referring to author, title of article, volume, and page number. Remit $3.00 for photocopy or $2.00 for microfiche.

(16) F. A. Long and W. F. McDevit, *Chem. Rev.*, **51**, 119 (1952).

(17) A more complete form of eq 5 is $\log f_i = k_s C_s + K_i(C_i^s - C_i^0)$ where K_i is the self-interaction constant. The second term here describes the contribution of self-interaction effects of the nonelectrolyte to the observed activity coefficients. This self-interaction term is considered to be negligible in all of the experiments reported here. This is reasonable since the compounds were present at low concentrations (<0.02 *M*) in all experiments. The fact that the distribution coefficients between water and the reference phases of all compounds (except AG_3E which was not examined) were independent of concentration is evidence against significant self-interaction effects.

Table II. Salting Out Constants (k_s) at 25.0° [a]

	AG_1E	AG_2E	AG_3E	AG_4E[g]	Formamide[b]	Acetamide	*N*-Methylacetamide
Na_2SO_4	0.56[c]	0.58[c]	0.55[c]	0.52[c]	−0.02[c,e]	0.20	0.36
NaCl	0.16	0.13	0.09[c]	0.05[d]	−0.04	0.05	0.11
NaBr	0.11	0.07	0.05	0.00	−0.03	0.03	0.08
NaI	0.04	−0.04	−0.13[c]	−0.22[c]	−0.04		
$NaClO_4$	−0.03[c]	−0.13[c]	−0.21[d]	−0.32[d]	−0.08	0.00	0.03
NaSCN	−0.03[c]	−0.10[c]	−0.21[d]	−0.26[d]	−0.09	0.00	0.01
$NaCl_3CCOO$	−0.02[c]	−0.08[c]	−0.17[c]	−0.23[c]			
KF	0.31	0.31	0.27	0.23	0.00		
KCl	0.15	0.12	0.07[c]	0.05[c]	−0.03	0.07	0.12
CsCl	0.14	0.10	0.05[c]	0.06[c]	−0.03	0.07	0.11
LiCl	0.10	0.07	0.04[c]	0.01	−0.03	0.03	0.05
$CaCl_2$	0.14	0.07	0.01[c,f]	−0.09	−0.10		
$(CH_3)_4NBr$	0.06	0.00[c]	0.00[c]	0.02[c]			

[a] The salting out constant is defined by eq 5. The values of k_s were determined as described in the text and are based on a range of salt concentrations of 0–2.0 *M* unless otherwise indicated. The values are rounded off to the nearest hundredth. [b] From experiments with ether as the reference phase. [c] Based on activity coefficients at salt concentrations of 1.0 *M* and below. [d] Based on salt concentrations of 0.5 *M* and below. [e] Plot markedly curved with average k_s value *ca.* −0.02 (see Figure 5). A value of *ca.* +0.02 is observed from experiments with *n*-hexanol as the reference phase. [f] There is salting out of AG_3E at $CaCl_2$ concentrations above 1.0 *M*. This behavior differs from that of the other three glycine peptides for which plots of log *f* against $CaCl_2$ concentration are approximately linear. The results with AG_3E could be accounted for by the precipitation of a $ClCl_2 \cdot AG_3E$ complex at high $CaCl_2$ concentrations but has not been investigated further. [g] Data from ref 6 recalculated.

and values which are based on initial slopes are indicated. The standard errors for the k_s values were determined for the linear plots and were all less than ±0.010, and in most cases less than ±0.006. The k_s values for Na_2SO_4 were based on salt concentrations of 1.0 *M* and below and were determined by computer. Standard errors of Na_2SO_4 values were slightly larger than those for other salts but were all less than ±0.016. The k_s values which are based on initial slopes (for salts other than Na_2SO_4) are obviously less accurate than those based on linear plots, but were not subjected to statistical analysis.

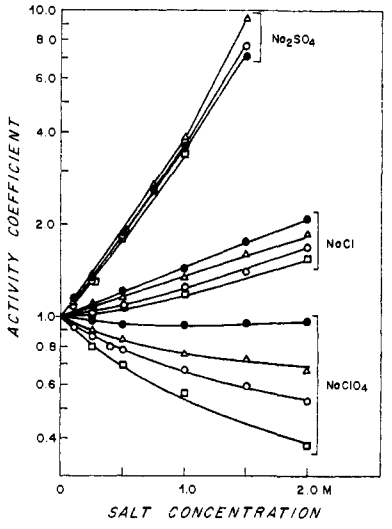
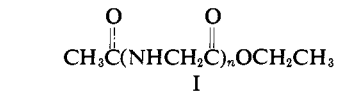


Figure 1. Activity coefficients of four glycine peptides in solutions of three salts at 25°. Symbols are: ●, AG_1E; △, AG_2E; ○, AG_3E; □, AG_4E.

Salt effects have been determined on three acetylglycine ethyl esters and previously reported data for AG_4E are also included for comparison. These four compounds differ in the number of glycyl or peptide groups which they contain (I, n = 1–4), and, therefore, differences between salt effects on these compounds should represent the salt effects on the peptide group. Some typical data are presented graphically in Figure 1. There is a wide range of effects on the four acetylglycine esters for different salts. Of the salts investigated, Na_2SO_4 causes the strongest salting out, but the effects of Na_2SO_4 are nearly the same on all four compounds. Sodium chloride salts out less strongly and the extent of salting out decreases slightly with an increasing number of peptide groups. With $NaClO_4$ the degree of salting in also increases with the number of peptide groups, and the differences between the four compounds are larger than with NaCl.



I

A comparison of most of the salts based on salting out constants is shown in Figure 2 for the four acetylglycine esters. Values of the salting out constants generally decrease as the number of peptide groups increases. The plots are generally linear which indicates that the salting out constants are an additive function of the number of peptide groups in these compounds. The slopes of these plots are a measure of the contribu-

Table III. Salt Effects on the Peptide Group at 25° [a]

	k_s	ΔF_{tr}[c]
Na_2SO_4	−0.013	−15 (1 *M*)
NaCl	−0.037	−60
NaBr	−0.037	−95
NaI	−0.087	−185
$NaClO_4$	−0.097	−180
NaSCN	−0.077	−170
$NaCl_3CCOO$	−0.070	−215
KF	−0.027	−40 (1 *M*)
KCl	−0.033	−55
CsCl	−0.033	−55
LiCl	−0.030	−80
$CaCl_2$	−0.077	−190
$(CH_3)_4NBr$	−0.013	−20 (1 *M*)

[a] The differences between values for acetylglycine esters differing by a single peptide group were determined; *i.e.*, AG_2E–AG_1E, AG_3E–AG_2E, and AG_4E–AG_3E. The averages of these three values for each salt are listed. [b] The k_s values are from the data in Table II. [c] The ΔF_{tr} values are for 2 *M* salt solutions unless otherwise indicated.

Table IV. Salting Out Constants at 0.5 and 40.0° [a]

	0.5°			40.0°					
	AG_1E	AG_2E	AG_3E	AG_1E	AG_2E	AG_3E	FA	AA	NMA
Na_2SO_4	0.58		0.54	0.54	0.56[b]	0.54	0.03[c]	0.22	0.36[b]
NaCl	0.18	0.11	0.09[b]	0.15	0.13[b]	0.09[b]	−0.03	0.05	0.09
NaSCN	−0.02	−0.10[b]	−0.21[b]	−0.04[b]	−0.10[b]	−0.16[c]		0.045	0.05
$NaClO_4$	0.00	−0.10[b]	−0.22[b]	−0.04[b]	−0.12[b]	−0.16[c]	−0.07	−0.01	0.02
KCl	0.16	0.09[b]	0.07[b]	0.15	0.10[b]	0.08[b]	0.02	0.06	0.12
LiCl	0.12	0.08	0.05	0.11	0.10	0.06		0.03	0.06

[a] Reference phases are as indicated in Table I. [b] Based on initial slopes, from data at 1.0 *M* and below. [c] Initial slopes from points at 0.25 and 0.50 *M*.

tions of each peptide group to the k_s values, and the average contributions of a peptide group to the k_s values were determined from the differences between the salting out constants for these four compounds, and are listed in Table III. The effects of the alkali halide

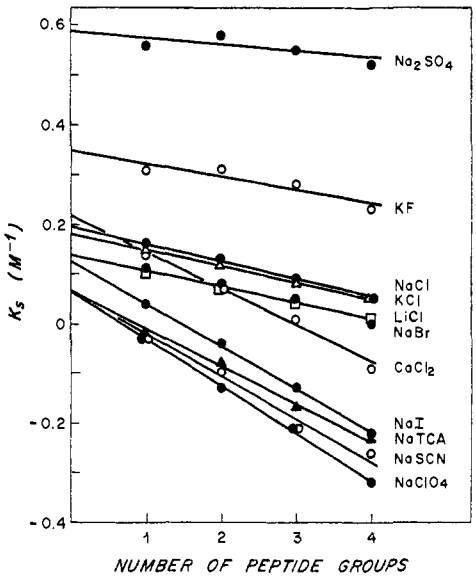


Figure 2. Salting out constants of glycine peptides at 25°. The number of peptide groups is equal to the number of glycyl groups. The k_s values are listed in Table II and the term is defined by eq 5. NaTCA is sodium trichloroacetate. Symbols are: ●, NaCl; △, KCl; □, LiCl; ●, NaBr; ○, NaSCN; and others are as indicated.

salts are all similar and decrease the salting out constants by about 0.03 M^{-1} for each peptide group and the effects of Na_2SO_4 and $(CH_3)_4NBr$ are smaller. The other salts result in a larger degree of salting in, decreasing the salting out constants by about 0.07–0.10 M^{-1} for each peptide group.

As a second basis for comparisons the free energies of transfer (ΔF_{tr}) have been calculated based on eq 6,

$$\Delta F_{tr} = RT \ln f_i \qquad (6)$$

where ΔF_{tr} represents the free energy of transfer of 1 mol of dilute solution of a given compound *i*, from water into salt solution at the same concentration in each solvent.[16,18] Therefore, negative ΔF_{tr} values represent a decrease in free energy in salt solutions, or salting in.

The values were calculated for 2.0 *M* solutions of most salts for the four acetylglycine esters and are shown graphically as a function of the number of peptide groups in these compounds in Figure 3. The average contributions of each peptide group to the ΔF_{tr} values are given in Table III. Values at 1.0 *M* are shown for three salts, for which data at 2.0 *M* are not available. In these cases, extrapolation of plots similar to those in Figure 1 indicates that approximate values at 2.0 *M* may be estimated by doubling the 1.0 *M* values, in order to compare all salts on an equimolar basis. The linearity of most of the plots in Figure 3 indicates that

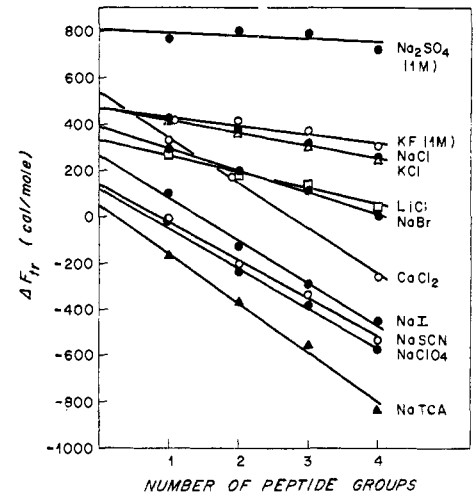


Figure 3. Free energies of transfer (ΔF_{tr}) of glycine peptides from water into 2.0 *M* salt solutions at 25°. Concentrations are 2.0 *M* unless otherwise indicated. Symbols are the same as in Figure 2.

the free-energy contributions of the peptide groups are generally additive, and the order of effects of different salts on the ΔF_{tr} values for peptide groups is similar to the order seen with k_s values. Therefore, both of these two parameters lead to similar conclusions.

The effects of a smaller series of salts on these compounds were investigated at 0.5 and 40° (Table IV). The temperature effects are illustrated for some representative salts in Figure 4, for the acetylglycine esters. There is a slight decrease in k_s values with increasing temperature for AG_1E and, as the number of peptide groups increases in these three compounds, there is a tendency for the temperature dependence of their k_s values to become reversed although the temperature effects are generally small. The peptide group contributions to k_s and ΔF_{tr} values were determined from the differences between these compounds, as described above for the data at 25°, and are tabulated in Table V. These results show a consistent decrease in the extent of salting in of the peptide group with increasing temperature over the 0–40° range. Therefore, the tem-

(18) In cases where plots based on eq 5 are linear, ΔF_{tr} and k_s values are directly related; $\Delta F_{tr}/RTC_s = k_s$. For nonlinear plots, the k_s values reflect effects of more dilute salt solutions.

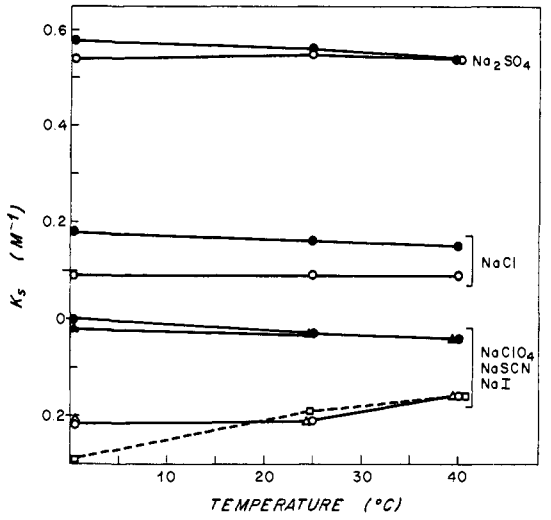


Figure 4. Effect of temperature on salting out constants of glycine peptides. Symbols are: ●, AG_1E; ○, AG_3E; ▲, AG_1E in NaSCN; △, AG_3E in NaSCN; □, AG_4E in NaI.

perature dependence of the salt effects on peptide groups is opposite to that of nonpolar compounds, although the temperature effects are small in both cases.[16] The salt effects on the peptide group also appear to be generally additive at 0 and 40°, within expected experimental errors.

Table V. Salt Effects on the Peptide Group at 0.5 and 40° [a]

	0.5°		40°	
	k_s	ΔF_{tr}[b]	k_s	ΔF_{tr}[b]
Na_2SO_4	−0.02[c]	−20[c]	0.00	5
NaCl	−0.045	−100	−0.03	−50
NaSCN	−0.10	−190	−0.06	−105
$NaClO_4$	−0.11	−235	−0.06	−120
KCl	−0.045	−90	−0.035	−40
LiCl	−0.035	−105	−0.025	−45[c]

[a] The values listed are averages of the differences between k_s and ΔF_{tr} values for the acetylglycine esters which differ by a peptide group; *i.e.* AG_2E–AG_1E and AG_3E–AG_2E. [b] All ΔF_{tr} values are for 2.0 *M* salt except for Na_2SO_4 which is 1.0 *M*. [c] $[AG_3E–AG_1E]/2$.

Salt Effects on Simple Amides. The distribution of formamide into nonpolar solvents is highly unfavorable, but satisfactory activity coefficient measurements were obtained with both diethyl ether and *n*-hexanol as reference phases. Two sets of experiments were performed with seven different salts using both hexanol and ether as reference phases. The results with the two reference solvents agree within experimental error, except for Na_2SO_4 which gave activity coefficients 5–10% larger with hexanol. As discussed in the Experimental Section, errors due to mutual solution of the phases should be more significant with ether than hexanol. The agreement between results with both solvents indicates that this is not a source of significant errors, with the possible exception of the Na_2SO_4 results. Even in the latter case the differences are not large.

The salt effects on formamide are nearly all in the direction of salting in (Figure 5, Table II). Even salts which have strong salting out effects on most organic compounds, Na_2SO_4 and KF, cause little or no salting out of formamide, although the k_s value for Na_2SO_4

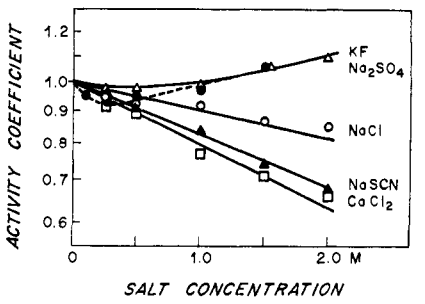


Figure 5. Activity coefficient of formamide in salt solutions at 25°. Symbols are: △, KF; ●, Na_2SO_4; ○, NaCl; ▲, NaSCN; and □, $CaCl_2$.

is difficult to determine because of the marked curvature of this plot. Comparison of the salting out constants of the three amides shows that the methyl groups in these compounds contribute large salting out effects for most salts (Tables II, IV). At 40° the salt effects on the amides do not differ significantly from those at 25° with the exception of NaSCN which gives slightly more salting out at 40° than 25°. The small differences shown between the effects of Na_2SO_4 at the two temperatures may be related to the different reference phases. Effects of Na_2SO_4 are essentially the same at both temperatures if experiments using hexanol as the reference phase are compared.

The data reported here for *N*-methylacetamide may be compared to previously reported work by Schrier and Schrier.[7] These authors reported a distribution coefficient for this compound between water and chloroform ($K_D = C_{ref}/C_{H_2O}$) of 0.0747 at 25°, over the concentration range 0.37–0.76 *M* in the aqueous phase. This is in excellent agreement with our value of 0.0748, obtained as the reciprocal of the value reported in Table I at the same temperature, although over a much lower concentration range. The two sets of data indicate that these distribution coefficients are independent of the concentration of *N*-methylacetamide over a concentration range of approximately 0.003–0.76 *M*. The salting out constants reported here for *N*-methylacetamide appear to agree with those reported by Schrier and Schrier within expected experimental error where the same salts were examined, with the possible exception of NaSCN. For this salt the previous value of −0.023 may be significantly more negative than our value of 0.01.

Discussion

The application of data from model compounds to proteins can be illustrated by considering the denaturation of a globular protein. In the native form the protein is largely excluded from the solvent and the protein becomes more exposed to the solvent in the denatured form. Therefore, any change in the solvent stabilizing the components of the protein which become exposed will favor denaturation. This model can be extended to include the aggregation of protein subunits and precipitation (salting out) since both will result in a progressive decrease in the degree of exposure of the protein to solvent. Similar models for denaturation have been used by many investigators and the study of model compounds to investigate the mechanisms of effects of urea, guanidine·HCl, and organic solvents, as well as salt solutions, has been reported previously

by others.[7,19] Comprehensive reviews of these subjects have recently appeared.[4,19f]

The experiments reported here with the acetylglycine esters provide reasonable estimates of salt effects on the peptide group, and it is also shown that peptide group effects are additive in these compounds. There are strong salting in effects by NaI, $NaClO_4$, NaSCN, $NaCl_3CCOO$, and $CaCl_2$; moderate salting in by KF, NaCl, KCl, CsCl, LiCl, and NaBr; and negligible or slight salting in effects by Na_2SO_4 and $(CH_3)_4NBr$. In order to further test the concept of group additivity in these compounds, their k_s values may be compared with those of ethyl acetate. These four compounds may be considered to consist of the elements of ethyl acetate plus one to four peptide groups. If the salt effects on these compounds represent the sum of effects on their peptide plus "ethyl acetate" portions, then extrapolation of the plots in Figure 2 to zero peptide groups should give the k_s values of ethyl acetate. The latter and the extrapolated values are given in Table VI

Table VI. Salting Out Constants of Ethyl Acetate and the Ethyl Acetate Component of Peptides at 25° [a]

Salt	Ethyl acetate[b]	Extrapolated from peptides[c]
Na_2SO_4	0.60[d]	0.59
NaCl	0.19	0.20
NaBr	0.12	0.14
NaI	0.02	0.12
NaSCN	0.02[d]	0.06
KF	0.30[e]	0.35
KCl	0.19	0.19
CsCl	0.15	0.17
LiCl	0.15	0.14
$CaCl_2$	0.26[d]	0.22

[a] Salting out constants are in units of M^{-1}. [b] A. P. Altshuller and H. E. Everson, *J. Amer. Chem. Soc.*, **75**, 4823 (1953). [c] The values are the ordinate intercepts of plots shown in Figure 3. [d] Reference 7. [e] Calculated assuming additivity of individual ion effects.

and it can be seen that there is good agreement, with the exception of NaI.

These compounds are considered to be appropriate models for the peptide group in the polypeptide chains of proteins. The data may be used directly to calculate the effects of salts on the free energy of an unfolded protein in which the entire polypeptide chain is exposed to the solvent. For example, the group of salts which strongly salt in the peptide group decreases the free energy of the peptide group by approximately 170–215 cal/mol in 2.0 *M* solutions. In completely exposed protein containing 100 amino acid residues, transfer into 2.0 *M* salt solutions of these salts will result in free-energy changes as large as −17 to −22 kcal/mol, from the peptide group effects of these salts. The alkali halides (except NaI) will have effects from one-third to one-half as large, and smaller but probably negative free-energy changes are calculated for Na_2SO_4 and $(CH_3)_4NBr$. Two kinds of additivity assumptions are made here. First, it is assumed that the contributions of the peptide groups are additive for the long polypeptide chains in ordinary proteins. The model compound experiments reported here provide some support for the additivity of peptide group effects in proteins, but this behavior could break down for large polypeptides. The second assumption is that the peptide group effects are independent of the amino acid side chains. Evidence is presented in the accompanying paper that salt effects on peptide groups and hydrocarbon side chains in amino acid derivatives are additive, supporting this assumption for proteins. Finally, we have defined the peptide group as the structure (CH_2CONH) which contains one more hydrogen atom than the repeating peptide unit of proteins, but the differences should be within the errors involved in our estimates.

It should be noted that we are only considering the contributions of peptide group effects to denaturation. The salting in, or decreases in free energy, of exposed peptide groups will usually be opposed by salting out effects, especially on nonpolar side chains. These salting out effects will tend to be smallest with salts having the strongest salting in effects on peptide groups and this will contribute to the strong denaturing effects of these salts. It is obvious that any attempt to calculate the salt effects on the free energy of denaturation or other changes in state of proteins would be a complex and difficult task. In terms of the model discussed above, the degree of exposure to the solvent of all of the components of the protein in both native and denatured forms can only be roughly approximated at the present time. The present experiments indicate that salts with strong denaturing effects cause large decreases in the free energies of exposed peptide groups in proteins, and these effects may account for denaturation by these salts.

Separation of Polar and Nonpolar Group Effects. Studies of salt effects on certain peptides, purines, and pyrimidines, and other polar compounds have shown that salt effects on these compounds differ largely from their effects on nonpolar compounds.[6,16,20] The observed effects on polar compounds may be considered to be the result of separate effects on their nonpolar and polar components but it is difficult to determine the contributions of individual groups experimentally to the observed overall effects. The differences between salt effects on polar and nonpolar compounds, especially in the order of effects of different salts, probably reflect different mechanisms. Little is known about the mechanisms of salt effects, especially on polar compounds, and attempts to separate the contributions from polar and nonpolar groups may help in understanding the mechanisms.

Schrier and Schrier have estimated salt effects on the amide (CONH) group from salting out constants on *N*-methylacetamide and *N*-methylpropionamide at 25°.[7] These authors assumed that the salt effects on these compounds could be divided into methyl, methylene, and amide group contributions which were independently additive. Salting out constants for the methylene group were considered to be equal to the differences between the salting out constants of the two compounds, and somewhat larger values were estimated

(19) (a) W. Kauzmann, *Advan. Protein Chem.*, **14**, 1 (1959); (b) C. Tanford, *J. Amer. Chem. Soc.*, **86**, 2050 (1964); (c) D. B. Wetlaufer, S. K. Malik, L. Stoller, and R. L. Coffin, *ibid.*, **86**, 508 (1964); (d) J. F. Brandts, *ibid.*, **86**, 4302 (1964); (e) D. R. Robinson and W. P. Jencks, *ibid.*, **87**, 2462 (1965); (f) C. Tanford, *Advan. Protein Chem.*, **24**, 1 (1970).

(20) D. R. Robinson and M. E. Grant, *J. Biol. Chem.*, **241**, 4030 (1966).

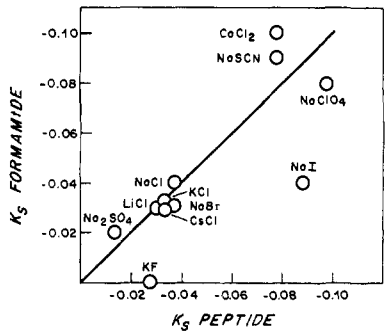


Figure 6. Comparison of the salting out constants for formamide and the peptide group at 25°. The line represents equal values.

for methyl groups. The methyl and methylene group effects were subtracted from the observed k_s values to give k_s values for the amide group. These results indicate that the amide group is salted in by all salts, with the exception of Na_2SO_4, which has no significant effect. Calculated salting out constants for the amide group were in the range of -0.10 to -0.20 for most salts, and the value for Na_2SO_4 was $+0.008$. In contrast, both amides are salted out by most salts, indicating that the effects on these compounds differ considerably from effects on the amide group.

The salt effects on formamide reported here should mainly reflect the amide group and may provide estimates of the contributions of the amide group to salt effects on peptides. Formamide is salted in by most of the salts but the salting out constants are only about one-half as large as the calculated values for the amide group of Schrier and Schrier, except for the Na_2SO_4 values which show negligible effects for both formamide and the amide group. Comparison of the three amides, formamide, acetamide, and *N*-methylacetamide, provides a measure of the contribution of methylene groups to salt effects. It may be noted that the differences between salting out constants of formamide and acetamide are significantly larger than differences between acetamide and *N*-methylacetamide for several salts (Tables II and IV), which indicates that hydrocarbon and amide group effects are not completely additive in these compounds. These data suggest that the additivity assumptions made by Schrier and Schrier may have led to significant errors.

In view of the large salting out contributions of the methyl groups of these amides, it may seem surprising that the salt effects on formamide and on the peptide group are nearly the same (Tables II–V). This similarity is shown in Figure 6, at 25°, in which most points lie close to the line indicating equal salting out constants, with the exception of NaI and possibly KF which salt in formamide significantly less than the peptide group. It might have been expected that the salt effects on the peptide group would be similar to effects on acetamide based on their structural similarity but there is much more salting out of acetamide than of the peptide group. A possible explanation for the similarity of salt effects on formamide and the peptide group is that the peptide methylene group may be largely masked in its location between two polar groups, and therefore both the peptide group and formamide results may primarily represent amide group effects. Some precedent for this suggestion may be provided by observations of Cohn and Edsall on the effects of the hydrocarbon components of some amino acids and their derivatives of the solubilities of these compounds in ethanol (S_A) and in water (S_0).[21] Aliphatic hydrocarbon side chains ending in methyl groups were found to regularly increase the solubility ratio, log S_A/S_0, by an average value of 0.49 per methylene group in a series of amino acids, hydantoins, and related compounds. In contrast, certain compounds with hydrocarbon chains of varying lengths terminating in polar, *e.g.*, amide, groups have nearly the same solubility ratios. Examples of the latter are asparagine and glutamine which have log S_A/S_0 values of -3.402 and -3.466, respectively. It was also found that the contribution of amide groups to these solubility ratios is variable depending upon the position of the amide group in the molecule.

There are few data available in the literature which can be used to provide this kind of detailed testing of the additivity of group effects for salt solutions, particularly for hydrocarbon components located between polar components. One pertinent example is a comparison of salt effects on acetylalanine ethyl ester and acetyl-β-alanine ethyl ester, from the accompanying paper.[8] The differences between the salting out constants of these two compounds and those for AG_1E measure the effects of a methylene group either as a side chain or located between polar groups. Although the methylene group contributions are small and cannot be determined accurately, there is a consistent trend toward less salting out for the β-alanine compound, suggesting that the methylene groups in acetyl-β-alanine ethyl ester are partially masked from salt effects. This interpretation is tentative because the differences involved are small. Furthermore, the k_s values of Na_2SO_4 for the alanine and β-alanine derivatives are essentially the same, indicating that there is no masking in this case, where it would be readily detected. A further reservation about this interpretation is that formamide contains two hydrogen atoms in addition to an amide (CONH) group. It is possible that these hydrogen atoms could contribute a salting out effect. However, in order to account for the similarity of the salt effects on formamide and the peptide group on this basis, the salting out contribution of two hydrogen atoms in formamide would have to be as large as the contribution of the methylene group, which seems unlikely. We conclude that we cannot determine the salt effects on the amide group with certainty but they are probably similar to the effects on formamide and on the peptide group.

It might have been anticipated that tetramethylammonium bromide would cause moderately strong salting in of the peptide group, but its observed effect is negligible. This salt is known to cause salting in of hydrocarbons in contrast to the salting out produced by alkali halides.[16,22] Its effect on formamide was not examined but it causes strong salting in of urea compared to NaCl, suggesting that the amide group should be salted in more strongly by tetramethylammonium bromide than NaCl.[23] Therefore, the former

(21) E. J. Cohn and J. T. Edsall, "Proteins, Amino Acids and Peptides," Reinhold, New York, N. Y., 1943, pp 205–212.
(22) W.-Y. Wen and J. H. Hung, *J. Phys. Chem.*, 74, 170 (1970).
(23) (a) W.-Y. Wen and C. L. Chen, *ibid.*, 73, 2895 (1969); (b) E. E. Schrier and R. A. Robinson, *J. Biol. Chem.*, 245, 2432 (1970).

salt might have been expected to salt in the peptide group more strongly than NaCl if the hydrocarbon and amide group effects were additive. The observed effects suggest that at least some of the expected salting in effects of tetramethylammonium bromide are masked in these compounds.

Mechanisms of Salt Effects. There is a large literature describing the effects of salts on nonelectrolytes and many theories have been proposed to account for these effects, but in spite of these efforts the mechanisms of the salt effects are poorly understood.[16,24] Even in the case of nonpolar compounds the situation appears to be unexpectedly complex. Many polar compounds including peptides contain both polar and nonpolar components and it seems likely that the salt effects result from a combination of different mechanisms, adding to the difficulty of interpreting these effects. We will briefly consider the principle theories of salt effects here in order to examine some possible mechanisms for these amide group effects.

Electrostatic Theory. The theory originally proposed by Debye and McAuley relates salt effects to the dielectric constants of nonelectrolyte solutions.[25] Their equation is given in eq 7 where ϵ is electronic charge,

$$\ln f_i = \frac{\delta_i \epsilon^2}{2kTD_0} C_j Z_j^2 / b_j \qquad (7)$$

k is Boltzman's constant, D_0 is the dielectric constant of water, and C_j, Z_j, and b_j are the molar concentration, valence, and radius of the ion j, respectively. The dielectric decrement δ_i is the effect of the nonelectrolyte on the dielectric constant of water and is defined by eq 8. According to eq 7, the sign of the activity

$$D = D_0(1 - \delta_i C_i) \qquad (8)$$

coefficient is determined by the dielectric decrement of the nonelectrolyte, and the magnitude of the salt effects, for either salting out or salting in, will be greater for polyvalent than monovalent ions. Therefore, the electrostatic theory predicts salting in only for compounds which increase the dielectric constant of water. Most nonelectrolytes decrease the dielectric constant of water and the predicted salting in of the electrostatic theory has rarely been tested. It has previously been pointed out that hydrocyanic acid is salted in more strongly by K_2SO_4 than by NaCl or LiCl in accord with electrostatic theory.[26] Salt effects on urea and the present results with formamide are of interest in this connection since both these compounds have negative dielectric decrements.[27]

The activity coefficient of urea is decreased by salts in the order $LaCl_3 > CaCl_2 > LiCl$, compared on the basis of molarity.[28] The direction and order of these effects are consistent with the electrostatic theory, but the results were not in good quantitative agreement with the Debye–McAulay equation and it was concluded that other mechanisms are probably important.[28] Recently, it has been shown that Na_2SO_4 has small activity coefficient effects on urea which are mostly in the direction of salting out.[23b] In contrast, both NaCl and two tetraalkylammonium bromides cause salting in,[29] and therefore the order of effects of these salts is not in accord with the electrostatic theory. Finally, it can be shown that formamide has a negative dielectric decrement based on its dielectric constant of 110 at 20°.[30,31] The order of effects of different salts on formamide reported here is also inconsistent with the electrostatic theory, because of both the differences between the effects of univalent salts and the lack of salting in by Na_2SO_4 in view of the salting in by other salts.

Recent improvements in the electrostatic theory have been given by Conway, *et al.*, accounting for dielectric saturation effects and the effects of the primary hydration layer of ions.[32] We have not attempted detailed calculations for the amide group effects but it appears unlikely that these modifications of the electrostatic theory will result in significantly different conclusions. Another recent modification of the electrostatic theory by Givon, *et al.*, includes the dielectric decrements of salts which are usually positive, as well as nonelectrolytes.[33] Their equation predicts only salting *out* for both hydrocyanic acid and formamide and therefore it is in poorer agreement with experiments for these compounds than other forms of the electrostatic theory. In addition, salting out constants which were calculated for benzene appear to represent little or no improvement over previous electrostatic theories.

We conclude that the electrostatic theory cannot account for salt effects on the simple polar compounds, urea and formamide, and that other mechanisms appear to be more important for salt effects on peptide and amide groups in model compounds and proteins.

Other Mechanisms of Salt Effects. The internal pressure theory of Long and McDevit is reasonably successful in predicting salt effects on nonpolar compounds, but it has been shown to break down with polar compounds including AG_4E, and for similar reasons, the internal pressure theory cannot account for salt effects on either formamide or the peptide group.[6,16] Recently, the scaled particle theory has been applied to salt effects on nonpolar compounds but this approach is not applicable to polar compounds.[34] Finally, it remains possible that salts may exert their effects on model peptides and on proteins through some modifications of the structure of water, other than those structural changes which might occur as a basis for the theories which have just been considered. There is little evidence to support this suggestion, however, and it was previously concluded that there is no overall correlation between the salt effects on several parameters which reflect the structure of water and the observed salt effects on proteins or polar model compounds.[4,6,20] Similarly, we have found no good correlations between salt effects on formamide or the peptide group and effects on the viscosity B coefficient, unitary partial molal ionic en-

(24) B. E. Conway, *Annu. Rev. Phys. Chem.*, 17, 481 (1966).
(25) P. Debye and J. McAulay, *Phys. Z.*, **26**, 22 (1925).
(26) J. T. Edsall and J. Wyman, "Biophysical Chemistry," Vol. 1, Academic Press, New York, N. Y., 1958, Chapter 5.
(27) See E. A. Harrington, *Phys. Rev.*, 8, 581 (1916), for dielectric constants of aqueous urea solutions. The dielectric decrement of formamide is discussed below.
(28) M. Sarnowski and B. Baranowski in "Electrolytes," B. Pesce, Ed., Pergamon Press, New York, N. Y., 1962, p 187.
(29) V. E. Bower and R. A. Robinson, *J. Phys. Chem.*, **67**, 1524 (1963).
(30) R. A. Robinson and R. H. Stokes, "Electrolyte Solutions," Butterworths, London, 1955, p 448.
(31) J. A. V. Butler, *J. Phys. Chem.*, 33, 1015 (1929).
(32) B. E. Conway, J. E. Desnoyers, and A. C. Smith, *Phil. Trans. Roy. Soc., Ser. A*, **256**, 389 (1964).
(33) M. Givon, Y. Marcus, and M. Shiloh, *J. Phys. Chem.*, **67**, 2495 (1963).
(34) W. L. Masterton and T. P. Lee, *ibid.*, 74, 1776 (1970).

tropy, self-diffusion of water, proton spin–lattice relaxation time, and the nmr chemical shift.

A possible mechanism for the salting in of formamide and the peptide group is a direct ion–amide group interaction to form soluble complexes according to eq 9,

$$\text{peptide} + \text{ion} \underset{}{\overset{K_c}{\rightleftharpoons}} \text{peptide}\cdot\text{ion} \qquad (9)$$

where $K_c = [P\cdot I]/[P][I]$. The results are consistent with this mechanism and approximate values of K_c equal to 0.1 M^{-1} for alkali halides and 0.25 M^{-1} for salts causing the strongest salting in. These values apply to both formamide and the peptide group. Similar complexes were previously suggested to account for the salting in of peptides, purines, pyrimidines, and salt effects on proteins. There is no convincing evidence that complexation with salts is the mechanism of salting in of peptides and similar polar compounds, but arguments in favor of this mechanism have been presented in detail elsewhere.[4,6,20]

The Effects of Salts on the Free Energies of Nonpolar Groups in Model Peptides[1]

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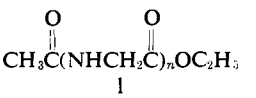
Abstract: The activity coefficients of *N*-acetylamino acid ethyl esters with hydrocarbon side chains (II) have been determined in aqueous solutions of several salts at concentrations up to 2.0 *M*. The derivatives of alanine, β-alanine, valine, norvaline, leucine, norleucine, and phenylalanine have been examined at 25° and those of alanine, norvaline, and norleucine at 0.5 and 40°. The salt effects on acetylglycine ethyl ester are subtracted from effects on the other compounds to give the contributions of the hydrocarbon side chains. The side chains are salted out by all of the salts examined and the extent of salting out is generally an additive function of the number of carbon atoms. Branching of the side chains has no significant effect. The salt effects on the hydrocarbon side chains of these model compounds are similar to the effects on these groups in simple hydrocarbons. This similarity indicates that hydrocarbon and peptide group contributions to salt effects on these compounds are additive. The results support the assumption that salt effects on peptide groups and nonpolar groups in proteins are independently additive. Values are obtained for the free energies of hydrocarbon amino acid side chains in salt solutions which should apply to proteins.

One approach to the investigation of mechanisms of salt effects on the structure and properties of proteins is to determine the effects of salts on model compounds which contain components of proteins. In the accompanying paper, the effects of several salts are reported on the activity coefficients of a series of four compounds which are models for peptide groups in proteins, I, where $n = 1$–4.[3] Comparison of these compounds, which differ in the number of peptide groups they contain, provides a measure of salt effects on peptide groups and indicates that the peptide group effects are additive. These results suggested that the contributions of peptide groups to salt effects on exposed proteins could be determined by simply multiplying the number of peptide groups by the effects on a single peptide group determined from these model compounds. This kind of calculation requires the assumption that the salt effects on amino acid side chains and on peptide groups are also additive, and it seemed desirable to investigate this assumption with further work on model compounds.



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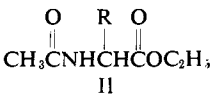

II

The location of nonpolar or other side chains in proteins in close proximity to neighboring peptide groups could conceivably modify salt or other solvent effects on either or both of these components. There is already a large literature on salt effects on the activity coefficients of polar and nonpolar nonelectrolytes, but questions of the additivity of group effects have received little attention in either polar or nonpolar compounds. We have investigated and report here the effects of salts on the activity coefficients of several *N*-acetylamino acid ethyl esters containing nonpolar side chains (II). These compounds are considered appropriate models for nonpolar side chains in proteins because the relationship of the side chains and neighboring amide and ester groups in the model compounds is similar to the relationship of side chains and peptide groups in proteins.

Experimental Section

Materials. The [^{14}C]-*N*-acetylamino acid ethyl esters were prepared according to the method described by Wolf and Nieman.[4] Amino acid ethyl ester hydrochloride (0.25 mol) was added to

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(4) J. P. Wolf, III and C. Niemann, *Biochemistry*, 2, 493 (1963).