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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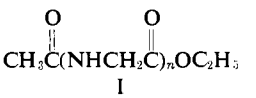
Abstract: The activity coefficients of *N*-acetylamino acid ethyl esters with hydrocarbon side chains (II) have been determined in aqueous solutions of several salts at concentrations up to 2.0 *M*. The derivatives of alanine, β-alanine, valine, norvaline, leucine, norleucine, and phenylalanine have 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.



I

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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(3) P. K. Nandi and D. R. Robinson, *J. Amer. Chem. Soc.*, **94**, 1299 (1972).

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

Table I. Distribution Coefficients between Water and Reference Phases[a]

Compd[b]	Reference phase	0.5°	25.0°	40.0°
Acetylalanine ethyl ester	Chloroform	0.367 ± 0.006 (7)[c]	0.230 ± 0.003 (10)	0.193 ± 0.003 (7)
Acetyl-β-alanine ethyl ester	Chloroform		0.332 ± 0.006 (10)	
Acetylnorvaline	Butyl ether	13.51 ± 0.60 (5)		2.29 ± 0.026 (6)
ethyl ester	Petroleum ether		20.24 ± 0.19 (7)	
Acetylvaline ethyl ester	Petroleum ether		19.08 ± 0.03 (6)	
Acetylnorleucine	Butyl ether	3.49 ± 0.09 (5)		0.600 ± 0.006 (6)
ethyl ester	Petroleum ether		4.75 ± 0.026 (8)	
Acetylleucine	Petroleum ether		7.27 ± 0.12 (9)	
ethyl ester	CCl_4		0.311 ± 0.008 (6)	
Acetylphenylalanine	Solid phase		3.41 ± 0.08 (13)[d]	
ethyl ester	Petroleum ether		5.04 ± 0.11 (5)	

[a] Distribution coefficients are C_i^0/C_i^r where C_i^0 is the concentration of compound i in water and C_i^r is the concentration in the reference phase. [b] All asymmetric compounds are of the L configuration. [c] The figures given are mean values ± the standard deviations and the number of determinations is in parentheses. [d] Solubilities in grams per liter.

NaOH (0.20 mol) in 100 ml of H_2O. 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 with Na_2CO_3 and the product was extracted with ethyl acetate. After removal of the ethyl acetate *in vacuo* the product was vacuum distilled.

***N*-Acetyl-β-alanine ethyl ester (AβAE)**[5] showed the following characteristics: bp 116–117° (1.1–1.2 mm); specific activity = 6.5 mCi/mol; ir, strong bands at 3458, 3003, 1728, 1671, 1518, 1408, 1278, 1213, 1045 cm^{-1}.

Anal. Calcd for $C_7H_{13}NO_3$: C, 52.81; H, 8.24; N, 8.80. Found: C, 52.80; H, 8.22; N, 8.90.

***N*-Acetyl-L-alanine ethyl ester (AAE)** showed the following: bp 110–112° (1.3–1.4 mm); mp 34–35° (lit. 34–35°)[6]; α^{20}_{589} −63.3 (*c* 5.2%, ethanol), α^{20}_{578} −64.3 (*c* 5.2%, ethanol) (lit. α^{20}_{578} −66.4° (*c* 6%, ethanol[6])); specific activity = 5.6 mCi/mol. The infrared spectrum showed strong bands at 3442, 3003, 1736, 1678, 1513, 1453, 1413, 1368 (m), 1338 (m), 1181, 1036 (m) cm^{-1}.

***N*-Acetyl-L-valine ethyl ester (AVE)** showed the following: bp 116–117° (1.0 mm); mp 33–35° (lit. 32.5–34.2°[7]); α^{25}_{589} −49.9° (*c* 4%, water) (lit. α^{25}_{589} −50.3° (*c* 4.5%, water[7])); specific activity = 4.8 mCi/mol. The infrared spectrum showed strong bands at 3440, 3013 (sh), 2975, 2948 (sh), 1738, 1680, 1514, 1413, 1260 (sh), 1223, 1180, 1039 cm^{-1}.

***N*-Acetyl-L-norvaline ethyl ester (AnVE)** showed the following: bp 115–116° (1.0–1.1 mm); mp 33–35°; α^{25}_{589} −58.5° (*c* 4%, water); specific activity = 4.5 mCi/mol. The infrared spectrum showed strong bands at 3435, 3000 (sh), 2965, 2938, 1727, 1668, 1503, 1403, 1255, 1217, 1175, 1038 cm^{-1}.

Anal. Calcd for $C_9H_{17}NO_3$: C, 57.73; H, 9.15; N, 7.48. Found: C, 57.74; H, 9.24; N, 7.53.

***N*-Acetyl-L-leucine ethyl ester (ALE)** showed the following: bp 116–117° (1.0 mm); α^{20}_{589} −43.15° (*c* 2.5%, ethanol) (lit. α^{20}_{589} −42.07° (*c* 2.7%, ethanol)[8]); specific activity = 9.0 mCi/mol. The infrared spectrum showed strong bands at 3447, 3012 (sh), 2974, 2947 (sh), 1737, 1679, 1514, 1409, 1302, 1221, 1181, 1041 cm^{-1}.

***N*-Acetyl-L-norleucine ethyl ester (AnLE)** showed the following: bp 117–118° (0.9–1.0 mm); α^{20}_{589} −29.7° (*c* 2.5%, ethanol); specific activity = 6.8 mCi/mol. The infrared spectrum showed strong bands at 3445, 3015 (sh), 2975, 2945, 2875 (m), 1738, 1680, 1515, 1413, 1270 (m), 1220, 1177, 1042 cm^{-1}.

Anal. Calcd for $C_{10}H_{19}NO_3$: C, 59.67; H, 9.52; N, 6.96. Found: C, 59.61; H, 9.53; N, 7.17.

***N*-Acetyl-L-phenylalanine ethyl ester (APE)** was recrystallized. A solubility phase curve was determined using 4.0-ml volumes of water at 25°, and revealed no impurity. Five determinations, in which the excess solid phase at equilibrium ranged from 5.3 to 37.4 mg, all gave the same solubility value within ±1%. *N*-Dibutyl ether was twice distilled, bp 141–142° (lit. 142°[9]). Chloroform and petroleum ether (bp 37.1–48.1°) were analyzed reagents and were used without further purification.

Radioactivity measurements were obtained with a Packard Model 3375 liquid scintillation spectrophotometer as described elsewhere.[3] All samples were corrected for sufficient periods to accumulate >20,000 net counts, reducing the statistical errors of counting to standard errors of <0.5%. Infrared spectra were obtained with a Perkin-Elmer Model 221 spectrophotometer.

Measurements of solubility and distribution coefficients were carried out by methods described in detail elsewhere.[3] Equilibration for distribution experiments were carried out for at least 5 hr at 0.5° and 1 hr at both 25 and 40°. It was shown that these periods of equilibration were adequate for equilibrium to be reached by measuring distribution coefficients at varying times of equilibration. These experiments were performed with all the compounds in 1.0 *M* Na_2SO_4, 1.0 *M* NaCl, and 2.0 *M* KCl at 25°; with AAE in 1.0 *M* Na_2SO_4 and with AAE, AnVE, and AnLE in 2.0 *M* NaCl at 40°; with AAE, AnVE, and AnLE in 1.0 *M* NaCl and 1.0 *M* KCl at 0.5°. Following equilibration of the compounds with solvents, the tubes were allowed to stand for about 4 hr at 25 and 40°, and overnight at 0.5° in order to allow separation of the phases. Samples were removed for counting radioactivity from both phases of each tube.

Activity coefficients of APE were determined from solubility measurements. Equilibrations were carried out for approximately 72 hr. It was established that equilibrium had been reached within this time period both by varying the time of equilibration and by approaching equilibrium from supersaturated and undersaturated solutions, in water and in the presence of four different salts. The concentration of APE was determined spectrophotometrically from the difference in absorbance at 257.5 nm (λ_{max}) and at 300 nm where the absorbance is small. The extinction coefficient of APE for the difference in absorption at these two wavelengths was found to be 1.88 × 10^3 M^{-1} cm^{-1} in water. Samples of the saturated solutions were diluted by factors of six to ten with water for absorbance measurements. This reduces the salt concentration to levels where no significant effects of salts on the ultraviolet spectrum would be expected. The absorbance ratios of a standard solution of APE, $A_{257.5}/A_{250}$ and $A_{257.5}/A_{270}$, were found to be the same in water and in the most concentrated solution of each salt, within experimental error. The extinction coefficient of APE at 257.5 nm was unchanged in 1.0 *M* Na_2SO_4.

The solubility of APE in water and the distribution coefficients of all other compounds between water and reference phases are given in Table I. In nearly every case all of the measurements fell within ±3% of the mean value for distribution coefficients. Solubility measurements were within ±5% of the mean value. We assume that these ranges of experimental error apply to determinations in salt solutions as well. It was also shown for all compounds that the distribution coefficients were independent of the nonelectrolyte concentration over an approximate tenfold range which included the range of concentration used in these experiments in every case.

(5) Abbreviations for the *N*-acetyl ethyl ester derivatives of the following amino acids are: AβAE, β-alanine; AAE, alanine; AVE, valine; AnVE, norvaline; ALE, leucine; AnLE, norleucine; APE, phenylalanine.

(6) K. Freudenberg and F. Rhino, *Chem. Ber.*, **57**, 1547 (1924).

(7) H. R. Waite and C. Niemann, *Biochemistry*, **1**, 250 (1962).

(8) S. Tsboyama, *Bull. Chem. Soc. Jap.*, **39**, 698 (1966).

(9) J. H. Mathews and P. R. Fehlandt, *J. Amer. Chem. Soc.*, **53**, 3212 (1931).

Table II. Activity Coefficients of Compounds Determined with Different Reference Phases at 25.0°

Ref Phase	$CHCl_3$				Petroleum ether			CCl_4			
	Salt concn, *M*										
	0.50	1.0	1.5	2.0	0.50	1.0	2.0	0.50	1.0	1.5	2.0
					Acetylalanine Ethyl Ester						
Na_2SO_4	2.16	4.62			2.11	4.49		2.20	4.61		
NaCl	1.25	1.59		2.36			2.41	1.25	1.57		2.40
KCl		1.55		2.49		1.58	2.47		1.57		
					Acetyl-β-alanine Ethyl Ester						
NaCl		1.52	1.85	2.20					1.48	1.81	2.18
					Acetylleucine Ethyl Ester						
Na_2SO_4					2.80			2.92			

As a check on the distribution experiments, occasional experiments were performed in which activity coefficients were determined in salt solutions using different reference phases. The results of these experiments are given in Table II, and they show that activity coefficients determined with different reference phases agree within experimental errors.

Results[10]

Activity coefficients were determined from solubility and distribution measurements using eq 1 and 2, respectively.[3] In these equations, f_i^s is the activity coefficient of the nonelectrolyte, i, in salt solution, C_i^0 and C_i^s are the molar concentrations of i in water and in a given salt solution, and $C_i^{r,0}$ and $C_i^{r,s}$ are concentrations in the reference phases corresponding to water and to salt solutions, respectively. It is assumed that the activity coefficient in water in all cases is equal to one.

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

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

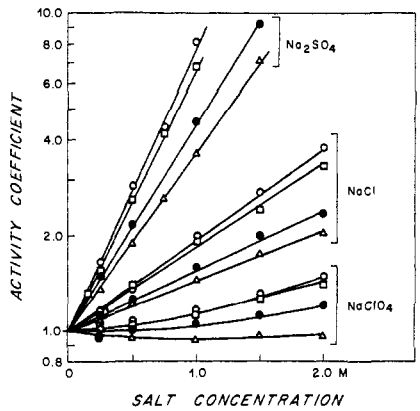

Figure 1. The effects of unbranched aliphatic side chains on the activity coefficients of model peptides in solutions of three salts at 25°. The compounds are: △, AG_1E; ●, AAE; □, AnVE; ○, AnLE.

The activity coefficients of nonelectrolytes in salt solutions usually follow the Setchenow equation (eq 3)

$$\log f_i = k_s C_s \quad (3)$$

where f_i is the activity coefficient of the nonelectrolyte, k_s is the salting out constant, and C_s is the molar salt concentration.[11,12] Most of the data reported here follow eq 3 throughout the range of salt concentrations examined. The salting out constants provide a convenient method of describing the salt effects and were calculated for all of the experiments. Values were obtained by regression analysis with the aid of a computer, for data which follow eq 3 over the entire range of salt concentration. Standard errors of the k_s values are all $<\pm 0.010$ and in most cases $<\pm 0.005$. The errors for Na_2SO_4 are somewhat larger but all are $<\pm 0.016$. Plots of log f against salt concentration for $NaClO_4$ and NaSCN usually showed significant curvature, and k_s values were estimated in the usual manner from initial slopes. For these two salts, initial slopes were approximated by passing a straight line visually through points over the range of 0–1 *M*, and these slopes were not subjected to statistical analysis. For Na_2SO_4 these plots were linear through 1 *M* salt and k_s values based on this concentration range were obtained in the usual manner by regression analysis.

Some typical results are shown graphically in Figure 1, in a semilogarithmic plot of activity coefficients as a function of salt concentrations based on eq 3. Compounds with methyl, *n*-propyl, and *n*-butyl side chains are compared to AG_1E in order to illustrate the contributions of aliphatic side chains of varying lengths to effects of these three salts. There is a wide range of effects on all of the compounds from the strong salting out by Na_2SO_4 to the weak effects of $NaClO_4$, and with each salt the degree of salting out increases with the size of the aliphatic side chains.

Salting out constants (k_s) for all salts at 25° for the compounds with unbranched aliphatic side chains are shown graphically as a function of the number of carbon atoms in the side chain in Figure 2. Data for AG_1E are included to give a series of four compounds which differ in the number of methylene groups in their side chains. For most of the salts, there is a linear increase in salting out constants, corresponding to increasing salting out, with increasing size of the side chains. The plots for NaBr and $NaClO_4$ appear to level off as the

(10) 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.

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

(12) The contribution of self-interaction effects of the nonelectrolytes to both k_s and ΔF_{tr} values is considered to be negligible for the following reasons. First, the concentration of the compounds in all of these experiments was less than 0.010 *M* in the aqueous phases for all distribution experiments and less than 0.015 *M* in all solubility experiments. Second, the distribution coefficients between water and reference phases for all of the compounds were found to be independent of concentration over approximately a tenfold concentration range, which included the highest concentrations present in all experiments.

Table III. Salting Out Constants (K_s) at 25.0° [a]

Compd	AG_1E [b]	AAE	AβAE	AnVE	AVE	AnLE	ALE	APE
Na_2SO_4 [c]	0.56	0.64	0.65	0.83	0.83	0.89	0.87	0.87
NaCl	0.16	0.19	0.18	0.26	0.26	0.29	0.28	0.27
NaBr	0.11	0.16	0.11	0.21	0.23	0.21	0.21	0.21
NaI	0.04	0.05	0.00					
$NaClO_4$	−0.03 [c]	0.02 [c]	−0.06 [c]	0.06 [c]	0.06	0.05 [c]	0.06	0.05
NaSCN	−0.03 [c]	−0.01 [c]	−0.06 [c]	0.00	0.02 [c]	0.00	0.01	
KF	0.30	0.35	0.33	0.46	0.44	0.49	0.48	0.43
KCl	0.15	0.20	0.17	0.25	0.26	0.28	0.29	0.24
CsCl	0.14	0.17	0.15	0.21	0.20 [c]	0.23	0.24	0.18
LiCl	0.10	0.14	0.11	0.19	0.19	0.22	0.22	0.21
$CaCl_2$	0.15	0.21	0.15			0.41	0.42	0.36

[a] Calculated as described in the text based on eq 3. [b] Data from ref 3. [c] Based on salt concentrations of 1.0 M and below.

Table IV. Salt Effects on Nonpolar Amino Acid Side Chains at 25.0° [a]

	k_s [b]				ΔF_{tr} [c]			
	AVE	ALE	APE	Av [d] $k_s/[CH_2]$	AVE	ALE	APE	Av [e] $\Delta F_{tr}/[CH_2]$
Na_2SO_4	0.27	0.31	0.31	0.085	370	410	435	130
NaCl	0.10	0.12	0.11	0.033	315	335	325	90
NaBr	0.12	0.10	0.10	*e*	320	260	250	*e*
$NaClO_4$	0.09	0.09	0.08	*e*	190	275	190	*e*
NaSCN	0.05	0.04		*e*	150	65		*e*
KF	0.14	0.18	0.13	0.050	340	460	330	115
KCl	0.11	0.14	0.09	0.033	285	375	235	105
CsCl	0.06	0.10	0.04	0.025	225	285	110	*e*
LiCl	0.09	0.12	0.11	0.030	250	320	295	85
$CaCl_2$		0.27	0.21	0.063		800	635	220

[a] The values are the differences between values for the compounds indicated and those for AG_1E. [b] From the data in Table III. [c] Calculated from eq 4 for 2.0 M salt solutions, except for Na_2SO_4 which is 1.0 M. [d] The average k_s or ΔF_{tr} per methylene group in the unbranched aliphatic side chains. The values for AAE, AnVE, and AnLE are divided by 1, 3, and 4, respectively, and the average of these three is taken for each salt. [e] The data indicate that the free-energy differences may not be an additive function of the number of CH_2 groups for this salt (Figures 2 and 3).

size of the side chains increases, and the effect of NaSCN is nearly the same on all four compounds. Salting out constants for all of the compounds at 25° are listed in Table III. The contributions of the nonpolar side chains to the k_s values are obtained by subtracting the k_2 values of AG_1E from those of the other compounds (Table IV). For the unbranched aliphatic side chains the average k_2 values per methylene group are calculated for salts whose effects are an additive function of the number of methylene groups, as shown in Figure 2.

As a further basis for comparisons, the free energies of transfer, ΔF_{tr}, from water into salt solutions, are calculated based on eq 4. Values at 25° are calculated

$$\Delta F_{tr} = RT \ln f_t \qquad (4)$$

for 2.0 M solutions of all salts except Na_2SO_4 which is 1.0 M. A reasonable estimate of the ΔF_{tr} values for 2.0 M Na_2SO_4 can be made by doubling the values at 1.0 M, since plots similar to those in Figure 1 were approximately linear for all compounds at Na_2SO_4 concentrations up to 1.5 M. The ΔF_{tr} values are presented similarly to the salting out constants and are conveniently summarized in Figure 3 for AG_1E and compounds with unbranched side chains. For most salts there is a linear increase in ΔF_{tr} with increasing size of the side chains. Exceptions are NaSCN which has nearly the same effect on all four compounds and NaBr and $NaClO_4$ which appear to level off with the larger side chains. It should be noted that the slope of the plot for Na_2SO_4 is based on 1.0 M solutions and would probably be about twice as large at 2.0 M. The side-chain contributions to ΔF_{tr} values are determined in a similar manner to their k_s values, and are given in Table IV.

Based on both k_s and ΔF_{tr} values the order of effects of different salts on the hydrocarbon side chains is listed

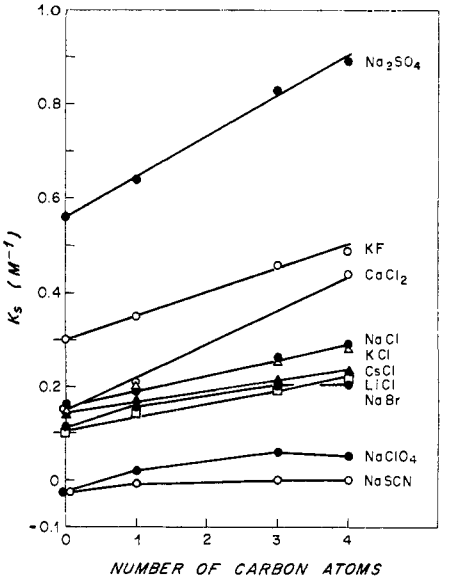

Figure 2. Salting out constants of acetylamino acid ethyl esters as a function of the number of carbon atoms in their unbranched aliphatic side chains at 25°. The compounds corresponding to 0, 1, 3, and 4 carbon atoms are AG_1E, AAE, AnVE, and AnLE, respectively. Symbols not clearly labeled are: △, KCl; ▲, CsCl; □, LiCl.

Table V. Salting Out Constants at 0.5 and 40.0°

	0.5°				40.0°			
	AG_1E[a]	AAE	AnVE	AnLE	AG_1E[a]	AAE	AnVE	AnLE
Na_2SO_4[b]	0.58	0.72	0.87	1.00	0.54	0.61	0.78	0.82
NaCl	0.18	0.21	0.29	0.31	0.15	0.18	0.24	0.28
$NaClO_4$	0.00	0.08	0.04[b]	0.13[b]	−0.04	0.01	0.01[b]	0.01[b]
NaSCN	−0.02	0.03[b]	0.06		−0.04	−0.01	−0.02[b]	−0.03[b]
KCl	0.16	0.21	0.26	0.33	0.15	0.18	0.24	0.26
LiCl	0.12	0.17			0.11	0.13	0.18	0.21

[a] Data from ref 3. [b] Values based on initial slopes of plots of log activity coefficient against salt concentration, using points at 1.0 M salt concentration and below. See text for details.

in the order of decreasing salting out: $Na_2SO_4 > CaCl_2 > KF > NaBr, NaCl, KCl, LiCl \geq NaClO_4 \geq CsCl > NaSCN, NaI$. This order is similar to the order of effects on nonpolar compounds generally, and the order for the phenylalanine side chain is in close agreement with the order of salt effects on benzene.[11] The order, as well as the absolute contributions for the larger aliphatic and the phenylalanine side chains, are quite similar but the latter appears to be more sensitive to changes in cations, mainly due to the weaker salting out by CsCl than other alkali metal chlorides. The isopropyl and isobutyl side chains of valine and leucine, respectively, have approximately the same effects as their corresponding unbranched side chains (Table III), and we conclude that chain branching makes no significant contributions to salt effects.

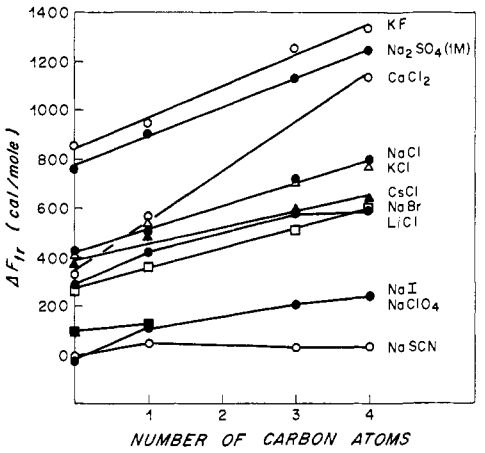

Figure 3. The free energy of transfer (ΔF_{tr}) of acetylamino acid ethyl esters from water into salt solutions at 25°, as a function of the number of carbon atoms in the side chains. All salt solutions are 2.0 M except Na_2SO_4. The numbers 0, 1, 3, and 4 refer to AG_1E, AAE, AnVE, and AnLE, respectively. The symbol, ■, is NaI, and others are as indicated in the legend to Figure 2.

The β-alanine compound was examined to determine whether salt effects on hydrocarbon components of these compounds are sensitive to the location of these groups. The alanine and β-alanine compounds each differ from AG_1E by a CH_2 group, but in the β-alanine compound it is located between two polar groups. For all salts except Na_2SO_4, the k_s difference values in Table III are smaller for the β-alanine than the alanine compounds. In most cases the differences between the two compounds are too small to be significant individually, but the consistently smaller values for the β-alanine compound indicate that the salting out contribution of the CH_2 group is partially masked in this compound by its location between polar groups.

Table VI. Salt Effects on Nonpolar and Amino Acid Side Chains at 0.5 and 40.0°[a]

	0.5°		40.0°	
	k_s	ΔF_{tr}[b]	k_s	ΔF_{tr}[b]
Na_2SO_4	0.114	155	0.065	100
NaCl	0.033	90	0.031	80
KCl	0.042	115	0.029	80
LiCl			0.023	60

[a] The values are the average k_s and ΔF_{tr} values per methylene (CH_2) group in unbranched aliphatic side chains. The results are based on results with AG_1E, AAE, AnVE, and AnLE (Table V), and are calculated as described in the footnotes to Table IV. [b] Salt solutions are 2.0 M except Na_2SO_4 which is 1.0 M.

The effects of temperature were investigated by examining a smaller series of salts on the three compounds with unbranched side chains, AAE, AnVE, and AnLE, at both 0.5 and 40° (Tables V and VI). The

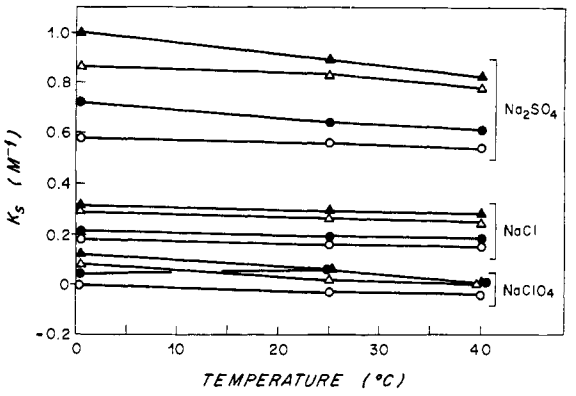

Figure 4. Salting out constants of four compounds at 0.5, 25, and 40°, for representative salts. Symbols are: ○, AG_1E; ●, AAE; △, AnVE; ▲, AnLE.

temperature effects are small and in the direction of decreasing salting out with increasing temperature as illustrated for three of the salts in Figure 4. The side chain contributions to the k_s and ΔF_{tr} values also show a tendency toward decreasing salting out with increasing temperature. The side-chain contributions to the salt effects are a linear function of the number of CH_2 groups in the side chains for Na_2SO_4, NaCl, and KCl at both 0.5 and 40°, and also for LiCl at 40°, as illustrated for k_s values in Figure 5. The effects of $NaClO_4$ and NaSCN are smaller, especially for the larger two side chains, and are probably not additive. This behavior is similar to the effects previously described at 25°.

Table VII. Contributions of Hydrocarbon Groups to Salting Out Constants of Hydrocarbons and Alcohols at 25° [a]

	Methanol[b]	Ethanol[b]	1-Propanol[b]	1-Butanol[b]	Av[c] $k_s/[CH_2]$	Peptides[d] $k_s/[CH_2]$
Na_2SO_4	0.154	0.249	0.336	0.417	0.088	0.085
	Methane[e]	Ethane[e]	Propane[e]	*n*-Butane[e]		
NaCl	0.127	0.162	0.194	0.217	0.030	0.033
LiCl	0.097	0.124	0.152	0.171	0.025	0.030
KI	0.098	0.103	0.101	0.097	0.000	~0.01[f]
			Biphenyl[g]	Benzene[h]	C_6H_4[i]	
Na_2SO_4			0.846	0.548	0.298	
NaCl			0.276	0.195	0.081	
KCl			0.255	0.166	0.089	
LiCl			0.218	0.141	0.077	
NaBr			0.209	0.155	0.054	
$NaClO_4$			0.113	0.106	0.007	
			C_6H_4[j]	CH_2[j]	C_6H_5CH[k]	C_6H_5CH
Na_2SO_4			0.298	0.088	0.386	0.31
NaCl			0.081	0.030	0.111	0.11
LiCl			0.077	0.025	0.105	0.11
			Diphenylmethane[b]	Benzene[h]		
Na_2SO_4			0.88	0.548	0.33[l]	0.31

[a] Salting out constants are in units of M^{-1}. Salting out constants for alkanes were extrapolated to 25° from values at 12.7 and 30°. The extrapolated values were slightly larger than the 30° values but the average k_s/CH_2 values were the same within 0.001. The data are in units of M^{-1} except for alkanes, which are based on molal salt concentrations. The differences between values based on the two concentration scales are insignificant for alkanes. [b] W. Rieman, III, *J. Chem. Educ.*, **38**, 338 (1961). [c] The average k_s/CH_2 is the average of the differences in k_s values for each of three pairs of compounds which differ by one CH_2 group. [d] From Table IV. [e] T. J. Morrison and F. Billett, *J. Chem. Soc.*, 3819 (1952). [f] From the difference between k_s values for AAE and AG_1E with NaI. [g] M. A. Paul, *J. Amer. Chem. Soc.*, **74**, 5274 (1952). [h] Reference 11. [i] The differences between k_s values for biphenyl and benzene. [j] From this table. [k] The sum of the values listed for the C_6H_4 and CH_2 groups. [l] The differences between the k_s values for diphenylmethane and benzene.

Discussion

One of the major aims of the work reported here is to provide information which will be useful in determining the contributions of nonpolar side chains to salt effects on the structure and properties of proteins. There is a large literature on the effects of salts on the activity coefficients of nonpolar compounds, but it is possible that hydrocarbons, for example, may not be appropriate models for hydrocarbon side chains in proteins. There is only a small amount of data in the literature which provide indications whether or not group effects are additive in hydrocarbons, and there has been little or no systematic study of polar nonelectrolytes to determine whether salt effects on hydrocarbon and polar groups in polar compounds are independently additive.

The order of effects on the compounds reported here and the accompanying paper changes with the composition of the compounds in a manner consistent with additivity of polar and nonpolar groups. This is illustrated by $CaCl_2$ which has one of the strongest salting in effects on formamide and the peptide group.[3] As the number of glycine residues decreases in the acetylglycine esters, the $CaCl_2$ effect changes from salting in to salting out, and the compounds with large hydrocarbon side chains reported here are strongly salted out, reflecting the large salting out effect of $CaCl_2$ on nonpolar compounds.[13] The effects of $NaClO_4$ and NaSCN change in the same direction as $CaCl_2$ with these compounds, but the salting out effects are always small, corresponding to the small salting out effects of these salts on nonpolar compounds.[11] The order of effects of different salts on the nonpolar side chains reported here is similar to that seen with nonpolar compounds, as pointed out in the Results.

A good test of the additivity of polar and nonpolar group effects is to compare the salting out constants for the nonpolar side chains of these compounds with salting out constants for these groups in hydrocarbons and other simple compounds. There are relatively small amounts of data in the literature on nonpolar compounds which are suitable for determining group contributions to salt effects, but some pertinent results are summarized in Table VII. In the upper portion of the table, salt effects on a series of alcohols and alkanes show that for these compounds the differences between the k_s values for successive members of each series are similar. The CH_2 group effects appear to be approximately additive and average

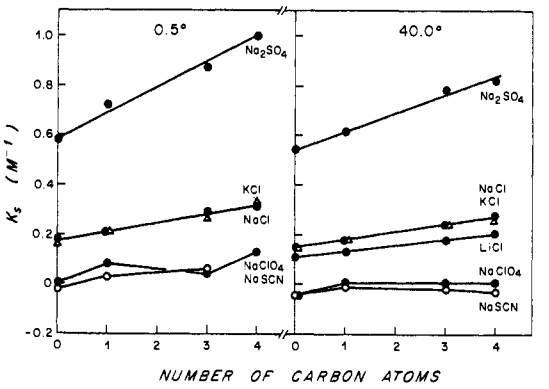

Figure 5. Salting out constants as a function of the number of carbon atoms in the side chains at 0.5 and 40°. See legend to Figure 2. The symbols are: △, KCl; ○, NaSCN.

(13) G. Akerlof, *J. Amer. Chem. Soc.*, **57**, 1196 (1935); see Table II in this reference.

values per CH_2 group are listed. The exception is KI which gives essentially the same k_s value for the four alkanes. The average values for Na_2SO_4, NaCl, and LiCl are in good agreement with the average CH_2 group contribution obtained from the side chains of the peptides.

The estimation of salt effects on the phenylalanine side chain (C_6H_5CH) in hydrocarbons is less straightforward. The differences between salting out constants of benzene and biphenyl are considered to represent a C_6H_4 group, and these values are added to CH_2 group effects determined from alkanes and alcohols in Table VII. The results are in good agreement with the phenylalanine side-chain k_s values given in Table IV for NaCl and LiCl. The value for Na_2SO_4 estimated in this way from hydrocarbons is significantly higher than that from the peptide side chain, but the value calculated from the difference between salting out constants for diphenylmethane and benzene is in good agreement. We conclude that the contributions of the alkane and the phenylalanine side chains to salt effects on the model peptides are similar to the contributions of these groups to salt effects on hydrocarbons for some salts. This indicates that the effects of these salts on polar and polar components of these peptides are independently additive. On the other hand, the effects of KI are essentially the same for the four alkanes, and $NaClO_4$ has about the same effects on biphenyl and benzene (and naphthalene, not shown here). These observations suggest that salts with the smallest tendency to salt out show a lack of hydrocarbon group additivity. This behavior appears to be in agreement with the apparent lack of additivity of $NaClO_4$, NaSCN, and possibly NaBr on the side chains, and is another similarity between the peptides and hydrocarbons.

It may also be pointed out that salting out constants for hydrocarbons examined here are often much larger than the k_s contributions of comparable groups in the peptide side chains or in hydrocarbons. For example, the k_s values of Na_2SO_4 and NaCl on butane and benzene are nearly twice as large as for the side chains of AnLE and APE, respectively. Salting out constants are approximately proportional to the molecular volume of hydrocarbons for these salts, considering compounds of comparable size.[14] From the molecular volumes of butyl and the $(CH_2)_4$ and C_6H_5CH groups estimated from parachor values, it can be shown that k_s values for the butyl side chains should be only *ca.* 10% smaller than butane and those for the APE side chain should be slightly larger.[15] Therefore, calculations of salt effects on hydrocarbon side chains of model compounds or on proteins should be based on differences between appropriate model compounds rather than on the salt effects on single compounds.

Mechanism of Salt Effects. The similarity of the effects of salts on hydrocarbon side chains of the model compounds to their effects on nonpolar compounds indicates that the same mechanisms operate in both cases. However, the mechanisms of the effects of salts on nonelectrolytes remain poorly understood, even for nonpolar compounds. The classical electrostatic theory is partially successful in accounting for salt effects on nonpolar compounds, but the predicted effects are generally too insensitive to the nature of the salt.[11] Recent improvements in the electrostatic theory have not solved this problem and the importance of electrostatic factors to salt effects on nonpolar compounds remains unclear.[16] A second approach is the internal pressure theory of Long and McDevit, which relates the salting out constant to the degree of electrostriction produced by the salt.[11] The major achievement of the internal pressure theory is its ability to predict the order of effectiveness of a wide range of different salts on nonpolar compounds, although somewhat arbitrary factors are required to obtain absolute k_s values in agreement with experimental results. Nonetheless, the internal pressure theory provides a reasonably satisfactory explanation for salt effects on nonpolar compounds.

Recently, the scaled particle theory has been applied to salt effects on nonpolar compounds. The theory has been formulated in an equation with two important terms.[17,18] One term describes the salting out effect which is based on the fact that salts generally increase the work required to create a cavity in the solvent. This is opposed by a salting in effect related to Van der Waal's interactions between the solute and the walls of the solvent cavity, and these interactions are generally stronger in the presence of salts. The net salt effects are determined by the sum of these two factors but are in the direction of salting out for small nonelectrolytes and alkali halides. It was shown by Masterton and Lee that in the case of small electrolytes, the predictions of the scaled particle theory are in reasonably good agreement with experiment.[18] Larger compounds, such as benzene, require an arbitrary factor to provide good agreement. It should be noted that this theory also predicts the order of effects of salts on benzene quite accurately. The testing has not been extensive, however, and in the most detailed investigation has been limited to alkali halide salts. Further use of this theory may be limited by the fact that results are highly sensitive to uncertainties in the diameters of anions and cations.[18] Whether the scaled particle theory is capable of predicting salt effects as satisfactorily as the internal pressure theory will require the application of the former to a wider range of different salts.

Application to Proteins. According to a model which is described elsewhere, protein denaturation, dissociation, and changes from the solid to solution phases are accompanied by increases in the degree of exposure of the protein to the solvent.[3] Therefore, these reactions will be favored by any change in solvent stabilizing the components of a protein which become exposed during the reactions. In principle, it should be possible to determine the effects of salts or other reagents on components of proteins from studies on model compounds containing these components. The results of experiments reported here, and in the accompanying paper, indicate that salt effects on the peptide and nonpolar groups in proteins are additive, and the results provide reasonable estimates of the contributions of these groups to the free energies of exposed proteins in salt solutions. At the present time the calculation of free-energy changes for these reactions of proteins re-

(14) N. C. Deno and C. H. Spink, *J. Phys. Chem.*, **67**, 1347 (1963).
(15) O. R. Quayle, *Chem. Rev.*, **53**, 439 (1953).
(16) B. E. Conway, *Annu. Rev. Phys. Chem.*, **17**, 481 (1966).
(17) R. A. Pierotti, *J. Phys. Chem.*, **69**, 281 (1965); S. K. Shoor and K. E. Gubbins, *ibid.*, **73**, 498 (1969).
(18) W. L. Masterton and T. P. Lee, *ibid.*, 74, 1776 (1970).

mains a difficult problem. In the case of denaturation, as an example, based on the above model, it is not easy to determine which components of a protein are exposed in both the native and denatured forms. Furthermore, model compound studies in salt solutions are not available for a majority of amino acid side chains and many approximations are necessary.

With these limitations in mind we have attempted to make some rough approximations of the effects of four different salts on the free energy of denaturation of bovine pancreatic ribonuclease. The contributions of only two types of groups are considered here, the nonpolar and peptide groups, and it is assumed that these groups will account for most of the effects. We will first calculate the contributions of these two components to the salt effects on a completely unfolded denatured form, assuming that in this form the protein is completely exposed to the solvent. Ribonuclease contains 124 amino acid residues[19] and the contributions of 123 peptide groups are obtained by multiplying the average peptide group ΔF_{tr} values from the accompanying paper by 123, and these values are reported in the second row of Table VIII. The contributions of nonpolar side chains are determined as follows, based on the data in Table IV. There are six Tyr and three Phe groups in ribonuclease which will be assumed to be equal to nine Phe groups. Other residues included are two Leu, three Ileu, nine Val, and twelve Ala groups, and the four Pro and four Met groups are taken to be equivalent to an additional eight Val residues.[19] The sums of the ΔF_{tr} values for these side chains are entered in Table VIII in the nonpolar group row. Residues which are neglected include several ionized side chains, but these will be largely exposed to solvent in both the native and denatured forms and therefore should contribute little to denaturation. In addition we have ignored 25 hydroxyl terminal (Ser, Thr) side chains and 17 with terminal amide groups (Asn, Gln), but the effects on these two types of groups should tend to cancel out. The total free-energy values in Table VIII may be considered to represent maximum contributions of peptide and nonpolar groups to denaturation. The actual contributions to denaturation will be some fraction of these values representing the difference in the degree of exposure of these groups to solvent in the native and denatured forms. Tanford has estimated that the fractions of exposed peptide and nonpolar groups in active and denatured globular proteins are approximately 0.4 and 0.75 for the two forms, respectively.[20] If this were the case for ribonuclease, the contributions to denaturation would be about one-third of the values estimated here.

Table VIII. Approximate Effects of Salts on the Free Energy of Nonpolar and Peptide Groups in Unfolded Ribonuclease at 25° [a]

	Na_2SO_4 1.0 *M*	NaCl 2.0 *M*	NaSCN 2.0 *M*	$CaCl_2$ 2.0 *M*
Nonpolar groups	+13,900	+11,100	+3,000	+22,500
Peptide groups	−1,900	−7,400	−20,900	−23,400
Total[b]	+12,000	+3,700	−17,700	−900
T_m^0 [c]	+12[d]	+1	−28	−24

[a] The contributions of nonpolar and peptide groups to the free energy of transfer of denatured bovine pancreatic ribonuclease from water into salt solutions in calories per mole, assuming that these groups are fully exposed to solvent. See text for further details. [b] Sum of nonpolar and peptide group contributions. [c] Change in midpoint of the transition temperature in these salt solutions, from P. H. von Hippel and K. Y. Wong, *J. Biol. Chem.*, **240**, 3909 (1965). The T_m is 61.5° in the absence of added salt. [d] $(NH_4)_2SO_4$.

Comparison of the calculated free-energy changes with the ΔT_m values for these salts indicates that the order of effects of these salts is in reasonable agreement with the calculations, except for $CaCl_2$. The salting in of peptide groups by $CaCl_2$ is approximately equal to the salting out of nonpolar groups, giving a value near zero, in contrast to the strong denaturing effect of this salt. This suggests that the random coil denatured state with maximal exposure of the protein to solvent may be less stable than a form in which the nonpolar groups are partly shielded, decreasing their unfavorable contact with this solvent. Similar calculations have previously been published by Tanford for organic denaturing agents and one inorganic salt solution, 3.0 *M* $CaCl_2$, based on more limited model compound data.[20] Tanford concluded that a random coil form of ribonuclease is not the most stable denatured form, and that the product of denaturation by $CaCl_2$ is a partly unfolded protein. Schrier and Schrier have calculated the transition temperatures for two proteins in salt solutions based on the Flory equation, but model compounds were limited to their own studies with two amides.[21] With the aid of several assumptions, satisfactory agreement with observed effects was obtained.

Finally, the salting out of proteins is probably best accounted for by effects of salts on nonpolar or slightly polar side chains. It cannot be readily accounted for by peptide or amide groups since neither are significantly salted out, even by Na_2SO_4. The pH independence of salting out constants of proteins indicates that charged groups are not a major factor.[22] The salting out constants of carboxyhemoglobin and β-lactoglobulin by Na_2SO_4 are 2.28 and 1.89 at 25 and 30°, respectively.[22,23] These values are approximately 6–7 times larger than the salting out constants of the leucine and phenylalanine side chains given in Table IV, and therefore the salting out of these proteins by Na_2SO_4 might be accounted for if precipitation removed the equivalent of about six–seven leucine or phenylalanine side chains from exposure to the solvent. These calculations for either denaturation or salting out are not intended to be taken very seriously because of the assumptions involved. However, they probably provide a measure of the more important interactions involved in denaturation and salting out of proteins.

(19) D. G. Smyth, W. H. Stein, and S. Moore, *J. Biol. Chem.*, **238**, 227 (1963).

(20) C. Tanford, *Advan. Protein Chem.*, 24, 1 (1970).
(21) E. E. Schrier and E. B. Schrier, *J. Phys. Chem.*, 71, 1851 (1967).
(22) A. A. Green, *J. Biol. Chem.*, **93**, 495 (1931).
(23) A. H. Palmer, *ibid.*, **104**, 359 (1934).