This equation may be differentiated and rearranged to represent the change in chemical potential of each component as a consequence of the addition of a small increment (dn_s) of salt to the system

$$\left(\frac{\partial \mu_{np}}{\partial n_s}\right)_{np,H_2O} = -\frac{n_s}{n_{np}}\left(\frac{\partial \mu_s}{\partial n_s}\right)_{np,H_2O} - \frac{n_{H_2O}}{n_{np}}\left(\frac{\partial \mu_{H_2O}}{\partial n_s}\right)_{np,H_2O}$$

We know that $\partial\mu_{np}/\partial n_s$ is positive, to varying extents for different ions, for the salting-out of nonpolar solutes. From thermodynamic "first principles," $\partial\mu_s/\partial n_s$ must be positive. Therefore, in order for the above equation to "balance," $\partial\mu_{H_2O}/\partial n_s$ *must* be negative, though not necessarily very large since $n_{H_2O}/n_{np} \gg n_s/n_{np}$. This requires that the added salt *lower* the chemical potential of the water, and in the limiting case (*e.g.*, the polystyrene columns), where all the water is within a few molecular diameters of a nonpolar surface, it is difficult to see how this can be achieved at the molecular level without the ions mixing with, and thus reorganizing, the structure of these water layers.

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Model Studies on the Effects of Neutral Salts on the Conformational Stability of Biological Macromolecules. III. Solubility of Fatty Acid Amides in Ionic Solutions†

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ABSTRACT: The solubilities of *n*-hexanamide, *n*-pentanamide, and *n*-butyramide in aqueous salt solutions are measured at several temperatures as a function of $NaClO_4$ and NaCl concentrations (these salts representing, respectively, a strongly destabilizing and an essentially "inert" perturbant of macromolecular stability). NaCl is found to be a more effective salting-out agent than $NaClO_4$ for all these amides, and thermodynamic parameters are derived for the transfer of each of these amides (at infinite dilution) from water to 1 M $NaClO_4$ or NaCl solutions. The free energy of transfer of a methylene group not directly adjacent to the amide dipole is shown to be a constant for each of these salt systems, corresponding to a free energy of transfer from water to 1 M $NaClO_4$ of $\sim$+60 cal/mol of CH_2, and a free energy of transfer from water to 1 M NaCl of $\sim$+100 cal/mol of CH_2. These values are approximately independent of temperature. Estimates are made for the (negative) free energy of transfer of an amide group from water to 1 M salt, and used to demonstrate that the average residue transferred from the interior of an average protein in a macromolecular unfolding process may be represented by a peptide group and $\sim$2 methylene units. It is also shown that *n*-hexanamide and perhaps *n*-pentanamide can be induced to form micelles at elevated temperatures and $NaClO_4$ concentrations.

In the preceding article (Hamabata and von Hippel, 1973), chromatographic measurements were reported which demonstrate that the mono-monovalent salts tested bind nonspecifically to the "ideal" amide dipole, and that the Hofmeister specificity of binding develops with the addition of methyl groups around the unsubstituted amide. It was also shown that the effects of these methyl groups on ion-binding specificity differ, depending on the exact amide attachment site. In this article we revert to classical solubility studies of a series of amides of structure $H_2NC(=O)(CH_2)_nCH_3$, using compounds with *n* ranging from two to four. These studies show that methylene groups further removed from the amide

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TABLE 1: Solubility of Hexanamide in Aqueous Salt Solutions at Several Temperatures[a]

	$NaClO_4$ Concn (M)						
T (°C)	0	0.5	1.0	2.0	3.0	4.0	6.0
6	0.161	0.136	0.125	0.099	0.083	0.072	0.032
16	0.203	0.195	0.180	0.149	0.116	0.091	0.053
25	0.258	0.240	0.230	0.194	0.164	0.137	0.077
29	0.275	0.265	0.265				
33	0.315	0.306	0.316	0.304	0.385	0.533	
35	0.325	0.369	0.390	0.464	0.612		
37	0.339	0.361	0.448	0.774			
41	0.389	0.491	0.888				

	NaCl Concn (M)		
T (°C)	0.5	1.0	2.0
6	0.134	0.095	0.054
16	0.154	0.115	0.065
25	0.214	0.147	0.083
37	0.262	0.208	0.141
41	0.309	0.237	0.157

[a] Solubilities are given in moles/liter. The $NaClO_4$ values listed represent the average of two or three different sets of experiments with a standard deviation of less than ±5%. The NaCl data represent single sets of determinations.

dipole, unlike the vicinal or "transitional" methyls, exhibit completely additive effects on the salting-out constants (or the free energy of transfer from water to various salt solutions) characterizing these model compounds. In addition it is shown that under some conditions the more nonpolar of these fatty acid amides can be induced to form micelles. The properties of these micelles will be discussed in the following article (Hamabata *et al.*, 1973).

Materials and Methods

Chemicals. The fatty acid amides used were *n*-hexanamide, *n*-pentanamide, and *n*-butyramide, and all were obtained from Eastman (Rochester, N. Y.) and used without further purification in these studies. Thin layer chromatography of the hexanamide in *n*-hexane revealed a minor component that did not move from the origin, and could correspond to *n*-hexanoic acid. Some solubility determinations were repeated with *n*-hexanamide recrystallized from cyclohexane. The solubilities were identical with those obtained with the nonrecrystallized material.

Reagent grade NaCl and $NaClO_4$ were obtained from Mallinckrodt and from Smith Chemical Co., respectively. All solutions were passed through 0.45-μ Millipore filters prior to use. All experiments were conducted in a "background" buffer of 0.01 M sodium cacodylate (Matheson Coleman and Bell) adjusted to pH 7.0.

Solubility Determinations. An excess of amide, together with 2 ml of salt solution, were placed in 8–12 ml total capacity vials fitted with Teflon-lined screw caps. The tubes were sealed with stretched vinyl plastic tape. Solubility equilibrium was obtained by shaking the tubes in thermostated (±0.5°) water baths for 5–8 days. To ensure that equilibrium had been reached, duplicate sets of tubes were preheated at 50–60° until all the solute had dissolved. Such supersaturated (with respect to amide) solutions were then equilibrated with the subsaturated (unheated) tubes at each experimental temperature. Equilibrium was considered to be established when the measured solubilities in the two sets of tubes differed by less than 1%.

Undissolved amides were removed by passage through 0.45-μ Millipore filters, and the filtrates were allowed to stand an additional 24 hr at the experimental temperature. All the hexanamide and pentanamide solutions showed no further change in measured solubility on standing, even after the addition of amide crystals to the filtrate. The butyramide filtrates generally yielded additional insoluble material on adding butyramide crystals, and so such a "renucleation," followed by a second filtration, was routinely performed on all the *n*-butyramide solutions. No further changes were detected after the second filtration.

Control experiments were carried out with and without the cacodylate buffer. The buffer had no effect on the measured solubilities in all the cases tested.

Assays. Generally 0.2-ml aliquots of the filtrates were removed and diluted 10–80 times with buffer, and amide concentrations were determined by absorbance at 230 nm. All the amides followed Beer's law over the entire experimental concentration range (0–0.05 M amide). Calibration curves were constructed for each amide on a weight basis.

Results

The solubilities of hexanamide, pentanamide, and butyramide in aqueous solutions of $NaClO_4$ and NaCl at various salt concentrations and temperatures are summarized in Tables I and II. The three amides are all salted-out by NaCl over the entire concentration and temperature range tested. On the other hand, the solubility of the amides in $NaClO_4$ varies markedly with both temperature and the length of the alkyl chain. Thus butyramide is slightly salted-in at all temperatures tested, while pentanamide and hexanamide are

TABLE II: Solubility of Pentanamide and Butyramide in Aqueous Salt Solutions at Several Temperatures.[a]

	Pentanamide							Butyramide						
	$NaClO_4$ Concn (M)				NaCl Concn (M)			$NaClO_4$ Concn (M)				NaCl Concn (M)		
T (°C)	0	0.5	1	2	0.5	1	2	0	0.5	1	2	0.5	1	2
6	0.553	0.531	0.500	0.408	0.444	0.362	0.264	1.96	1.96	2.00	1.94	1.75	1.60	1.26
16	0.636	0.633	0.621		0.518	0.450	0.295	2.19	2.29	2.35	2.37	2.06	1.83	1.45
25	0.788	0.813	0.797	0.762	0.637	0.580	0.357	2.64	2.87	3.02	3.23	2.44	2.15	1.66
37	1.108	1.320	1.523	2.12	0.866	0.679	0.466							

[a] Solubilities are given in moles/liter. The butyramide data represent the average of two or three sets of experiments with a standard deviation of less than ±5%. The pentanamide data are the results of a single set of determinations.

salted-out at low temperatures. However, at a critical temperature (~33° for hexanamide and ~25° for pentanamide) this behavior is sharply reversed and at higher temperatures these two fatty acid amides are *salted-in*. These phenomena are illustrated in Figures 1 and 2, where the solubilities of hexanamide at various temperatures are plotted as a function of $NaClO_4$ and NaCl concentration, respectively.

It is apparent (Figure 1) that the solubility of hexanamide in $NaClO_4$ decreases with increasing salt concentration from 6 to 29°, while above 33° the solubility increases sharply. In contrast, the solubility of hexanamide in NaCl decreases monotonically with salt concentration at all temperatures, though Figure 2 suggests that this decrease is less precipitous at the higher temperatures.

The switch from salting-out to salting-in at a critical temperature in the hexanamide–$NaClO_4$ system is more clearly visualized in Figure 3, where we plot the solubility of hexanamide *vs.* temperature for 0, 1, and 2 M $NaClO_4$. This abrupt change in solubility behavior at a given temperature for long molecules with polar "head" and nonpolar "tail" groups is called a Kraft point (*e.g.*, see Mukerjee, 1967) and is characteristic of the onset of micelle formation in such compounds (*i.e.*, it indicates that the critical micelle concentration, or cmc, has been reached). The properties of these micelles, and

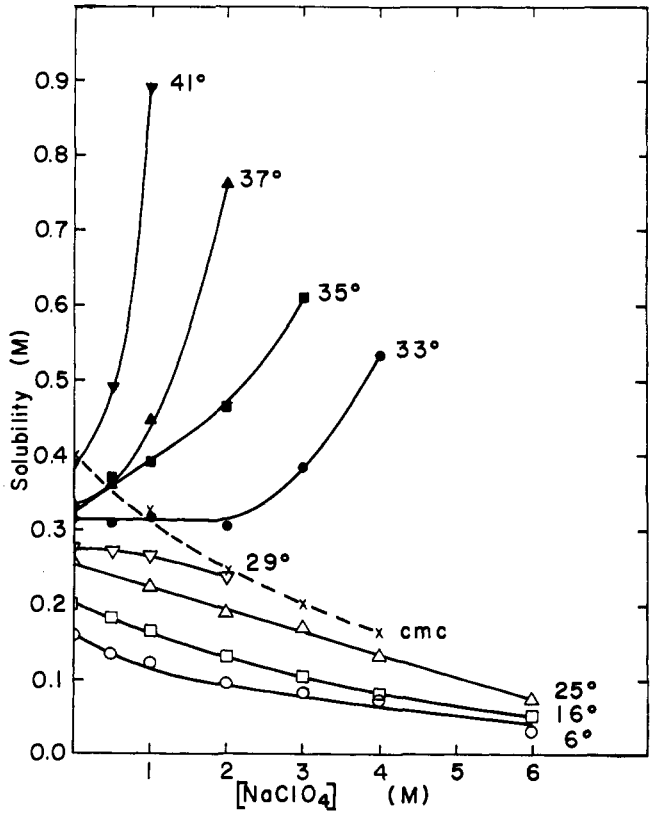

FIGURE 1: Solubility of *n*-hexanamide at various temperatures as a function of $NaClO_4$ concentration. The dashed line indicates the phase boundary between hexanamide monomers (below line) and hexanamide micelles (above line).

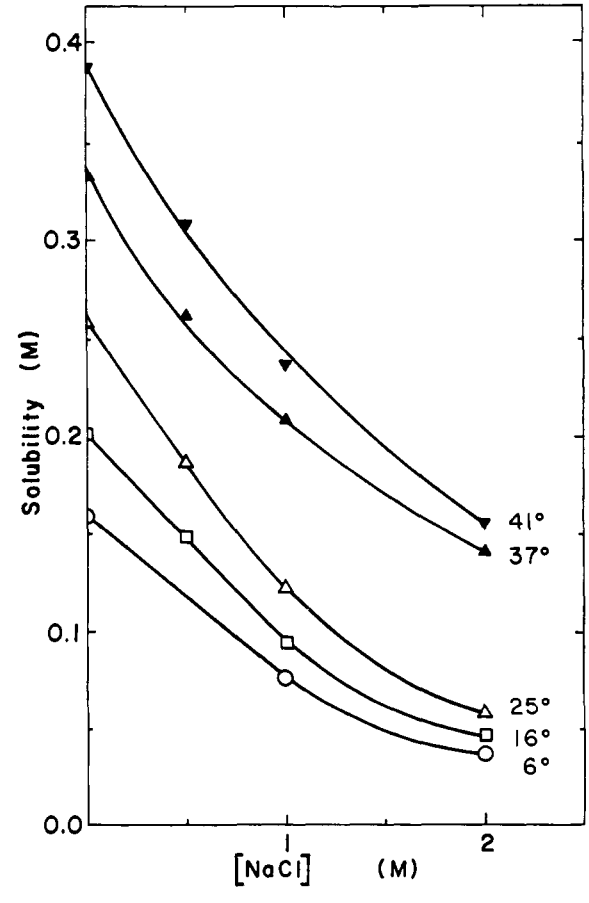

FIGURE 2: Solubility of *n*-hexanamide at various temperatures as a function of NaCl concentration.

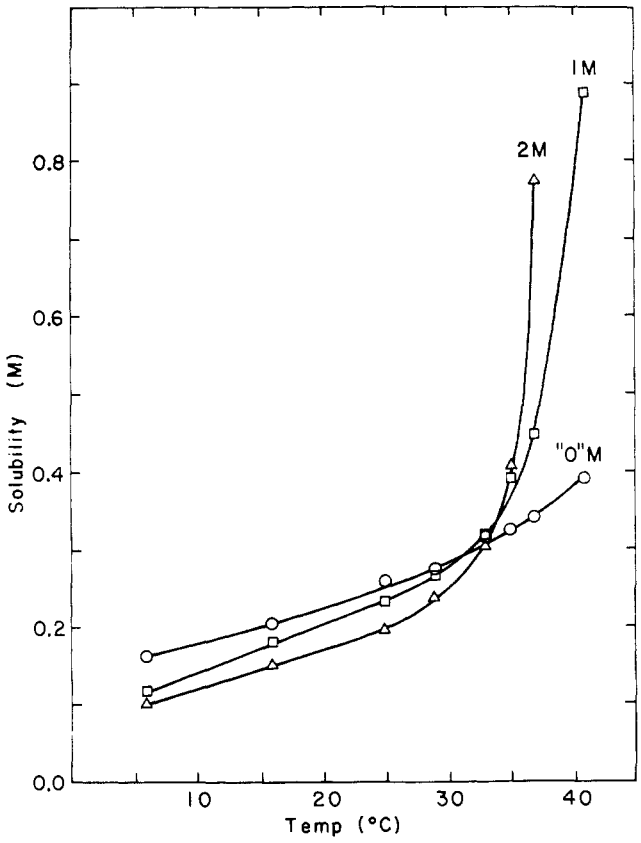

FIGURE 3: Solubility of *n*-hexanamide in "0" (buffer), 1, and 2 M $NaClO_4$ solutions as a function of temperature.

their behavior as a function of salt concentration and salt type, are discussed in the following article (Hamabata *et al.*, 1973). However, to confirm the above interpretation, the cmc for hexanamide (determined by fluorescent dye techniques) as a function of $NaClO_4$ concentration is also plotted in Figure 1. Clearly the cmc cuts through the data at exactly the point at which the solubility discontinuity occurs. In the rest of this article we will concern ourselves only with the pre-cmc solubility behavior of these amides.

As indicated in the previous article (Hamabata and von Hippel, 1973), activity coefficients may be defined for a particular electrolyte (s) as a function of salt concentration as

$$f_i = \frac{S_i^0}{S_i^s} \quad (1)$$

where S_i^0 and S_i^s represent the solubility of the amide in water (buffer) and salt (type s), respectively. f_i is defined as the molar activity coefficient of amide (i) in the salt solution, with the activity coefficient of the amide in water defined as unity. The salting-out constant, K_s, a parameter characteristic of the effects of a particular salt on the solubility of a nonelectrolyte in aqueous solution, is defined by the Setschenow equation

$$\log f_i = K_s C_s \quad (2)$$

where C_s is the concentration of salt (of type s). Values of K_s are defined by the initial slopes of plots of $\log f_i$ *vs.* C_s. Such plots for hexanamide, pentanamide, and butyramide at various temperatures as a function of $NaClO_4$ and NaCl are presented as Figures 4a and b through 6a and b. It is apparent that most of these plots are approximately linear for salt concentrations up to 2M, and these parts of the plots have been used to derive the values of K_s listed in Table III.

TABLE III: Salting-Out Constants (K_s).[a]

Salt	T (°C)	HA	PA	BA	$(\Delta K_s)_{CH_2,av}$
$NaClO_4$	6	0.093	0.040	0	+0.046
	16	0.053	0.010	−0.035	+0.044
	25	0.037	−0.012	−0.062	+0.050
	29	0.013			
	33	0.004			
	35	−0.071			
	37	−0.116	−0.140[b]		
	41	−0.203[b]	−0.282[b]		
NaCl	6	0.228	0.164	0.086	+0.071
	16	0.240	0.170	0.090	+0.075
	25	0.243	0.170	0.097	+0.073

[a] K_s is the Setschenow salt effect parameter calculated according to eq 3, using initial slopes of plots of f_i *vs.* salt concentration, up to 2 M salt. K_s is in units of M^{-1}. HA, hexanamide; PA, pentanamide; BA, butyramide. [b] The Setchenow plots are appreciably curved under these conditions. The initial slopes (and thus K_s) were estimated at salt concentrations of 0.5 M.

A number of qualitative observations may be derived from Figures 4a and b through 6a and b.

(1) The activity coefficient for *n*-hexanamide > 1.0 (hexanamide is salted-out) for all $NaClO_4$ concentrations (up to 6 M) at temperatures below 33°. At higher temperatures the cmc is crossed and hexanamide starts to associate into micelles (Figure 4a). This results in strong apparent salting-in, and a value of $f_i < 1.0$. Hexanamide is salted-out by NaCl at all concentrations up to 2 M, and temperatures at least to 41°. However, hexanamide seems to be appreciably less effectively salted-out by NaCl at 37 and 41° than at lower temperatures (Figure 4b).

(2) *n*-Pentanamide behaves similarly to hexanamide in both salts, though the temperature at which f_i changes from salting-out (>1.0) to salting-in (<1.0) in $NaClO_4$ comes at about 25° (Figure 5a). We have not demonstrated directly that this change in f_i for pentanamide corresponds to micelle formation, though this assumption is consistent with the properties of hexanamide and the progression in behavior from hexanamide to butyramide. NaCl salts-out pentanamide at all temperatures and salt concentrations tested. Again a clear, though less pronounced, discontinuity is observed between 25 and 37° (Figure 5b).

(3) *n*-Butyramide is salted-in by $NaClO_4$ at all temperatures above 6°, with no evidence of an abrupt discontinuity which might be attributed to micelle formation (Figure 6a). We may assume that this is because the alkyl tail of butyramide is too short to lead to stable micelle formation, and also that the solubility of butyramide is sufficiently high so that any potential favorable free-energy change which might be achieved by putting more butyramide into solution as micelles is more than offset by the entropy loss per monomer resulting from micelle formation. NaCl salts-out butyramide at all temperatures and salt concentrations tested (Figure 6b).

Values of K_s are summarized in Table III for all conditions

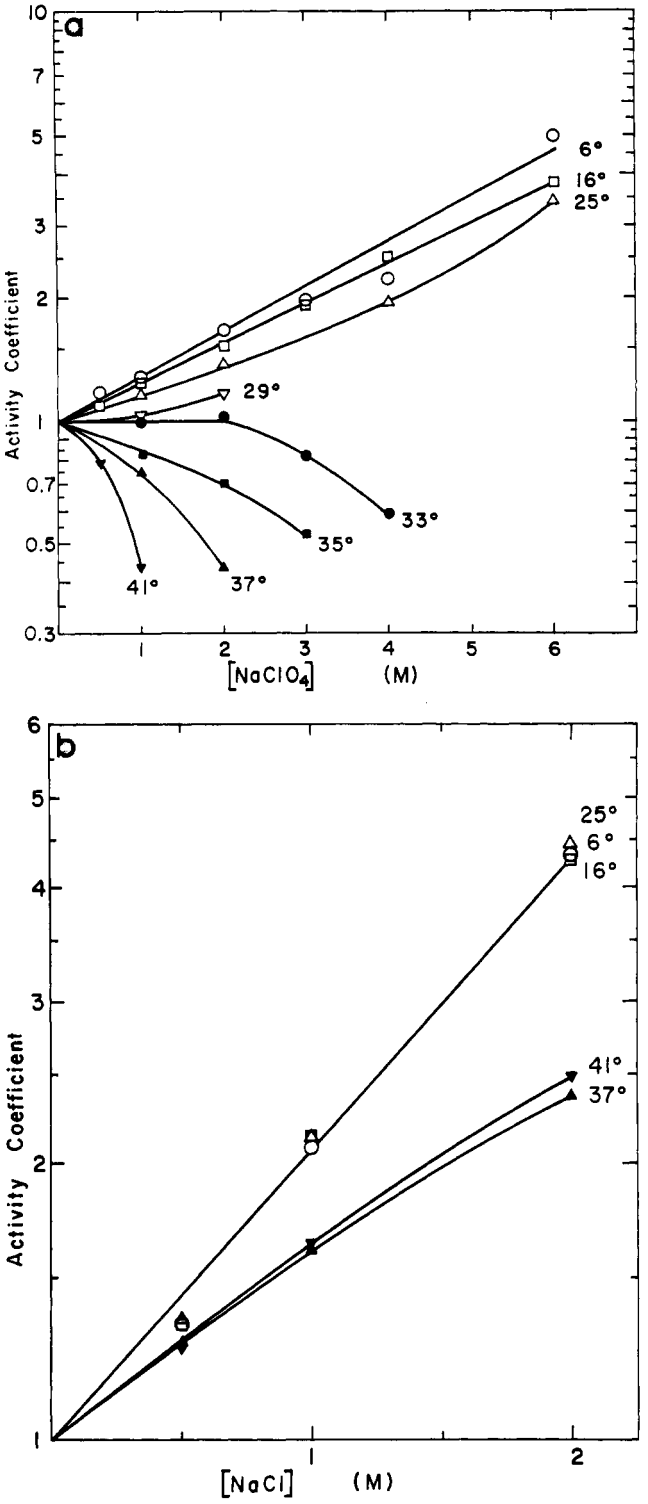

FIGURE 4: Activity coefficient (log scale) of *n*-hexanamide at various temperatures as a function of (a) $NaClO_4$ concentration; (b) NaCl concentration.

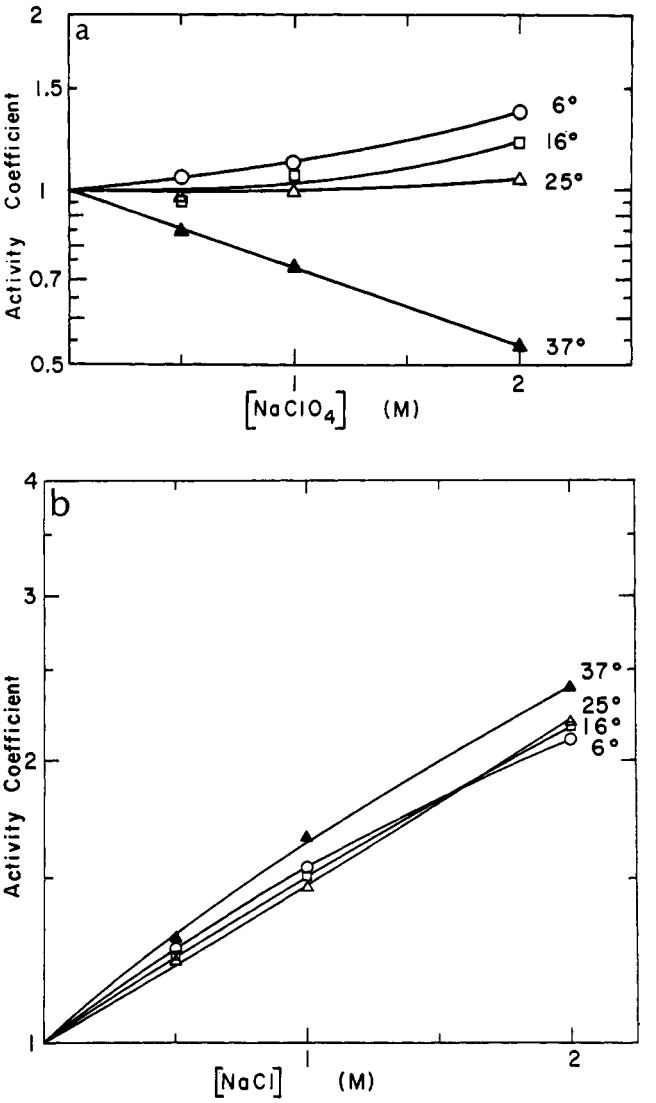

FIGURE 5: Activity coefficient (log scale) of *n*-pentanamide at various temperatures as a function of (a) $NaClO_4$ concentration; (b) NaCl concentration.

under which micelle formation was *not* observed. (Apparent K_s values for situations in which micelles are formed would correspond partially to the process of transferring amide from the solid form to micelles, and thus would be difficult to interpret unambiguously; however, see the next article in the series.) Average ΔK_s values corresponding to the *differences* between the apparent K_s values for hexanamide and pentanamide, and for pentanamide and butyramide, are also listed in Table III. These represent the K_s per methylene group, assuming the total K_s value represents the algebraic sum of the individual K_s values for the various functional groups of the amide molecule (Schrier and Schrier, 1967). The constancy of ΔK_s for hexanamide minus pentanamide, and for pentanamide minus butyramide, strongly supports this assumption, at least for methylene groups more than one carbon removed from the amide dipole (see Hamabata and von Hippel, 1973, for the somewhat contrasting behavior of the "transitional" methyl groups studied by binding methods).

In Figure 7 we plot log f_i *vs.* salt concentration for hexanamide, pentanamide and butyramide in $NaClO_4$ and NaCl at 25°. This graph demonstrates particularly clearly that the Hofmeister order is maintained for these systems (*i.e.*, ClO_4^- is much less effective than Cl^- in salting-out all the amides), and also shows the increment in salting-out effectiveness for each salt as the length of the alkyl chain is increased (*i.e.*, that increasing the nonpolar–polar ratio of the nonelectrolyte increases progressively the extent to which the nonelectrolyte is salted-out).

Finally, Figure 8 shows the behavior of K_s for the hexan-

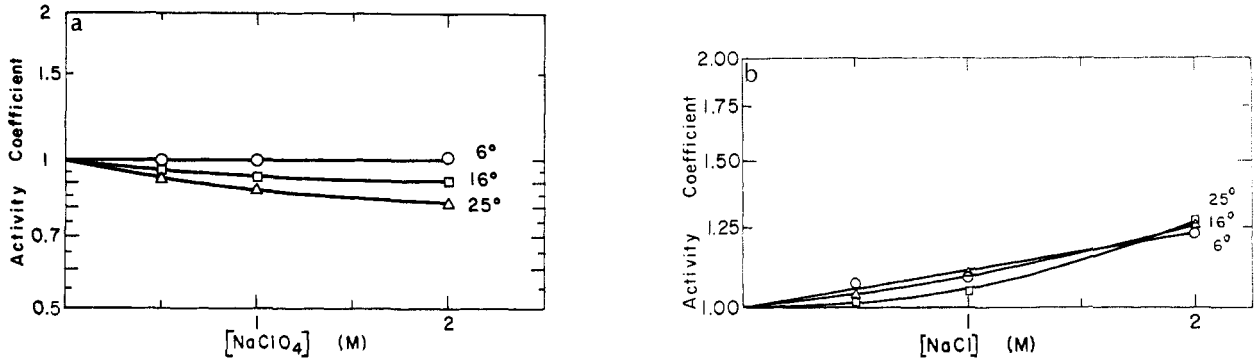

FIGURE 6: Activity coefficient (log scale) of *n*-butyramide at various temperatures as a function of (a) $NaClO_4$ concentration; (b) NaCl concentration.

amide–$NaClO_4$ system as a function of temperature. We may note that micelle formation occurs abruptly at about 33°, and that the data follow two straight lines.

The latter observation also bears on an assumption implicit in the way the K_s values were derived above. It has been pointed out by Long and McDevit (1952) and Robinson and Jencks (1965) that the complete Setschenow equation should contain a self-interaction term

$$\log f_i = \log \frac{S_i^0}{S_i^s} = K_s C_s + k_i(S_i^s - S^0) \qquad (3)$$

where K_s is the corrected salting-out constant, an self-interaction coefficient (for amide of type *i*). The magnitude of the self-interaction term depends upon the *difference* in solubility of the amide in water (buffer) and in the salt solution under test. We have not attempted to correct our data for self-interaction effects, since there are a number of reasons to think these effects will be small in those systems under pre-cmc conditions, where all the K_s measurements used in Table III were obtained.

(1) The solubilities of the amides tested change relatively little (less than a factor of two) with salt concentration at any particular temperature for most of the salt–amide systems examined (Tables I and II).

(2) The solubility of hexanamide in $NaClO_4$ changes by about a factor of two with temperature before the cmc is reached, and yet the apparent K_s (based on eq 2) shows a straight-line dependence on temperature (Figure 8), suggesting relatively negligible amounts of pre-cmc association between monomers. A variety of evidence for other micelle-forming systems suggests also that pre-cmc association is usually relatively limited (see Mukerjee, 1967).

(3) Association by interamide hydrogen bonding in aqueous solution has been shown to be negligible, even in much more concentrated amide solutions (*e.g.*, Klotz and Franzen, 1962).

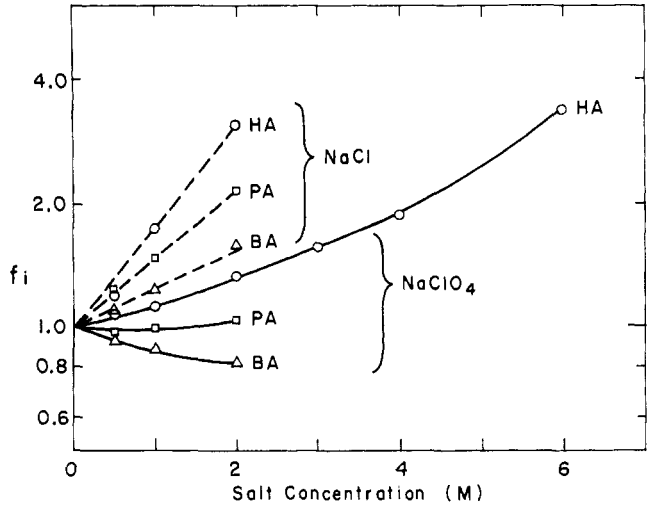

FIGURE 7: Activity coefficient (log scale) of hexanamide, pentanamide, and butyramide at 25° in NaCl and $NaClO_4$ as a function of salt concentration.

Discussion

In the preceding article (Hamabata and von Hippel, 1973) it was shown that the assumption of Schrier and Schrier (1967) that all nonpolar groups (methyl or methylene) have an equivalent effect on the salting-out constant of a nonelectrolyte (or alternatively, an equivalent modulating effect on salt binding to the amide dipole) is not totally correct, at least for methyl groups attached directly to the amide dipole. Similar conclusions have been reached by Nandi and Robinson (1972a,b) on the basis of solubility measurements on small amides. In this paper we demonstrate that the additivity of methylene group contributions to K_s (and thus to the free energy of transfer of the amide from water to salt solutions, see below) is restored for methylene groups further removed from the amide dipole.

We have compared the effects of various concentrations of $NaClO_4$ and NaCl on the solubility of *n*-hexanamide, *n*-pentanamide, and *n*-butyramide at several temperatures. The salts were chosen to represent two classes of effectors of the stability of biological macromolecules: $NaClO_4$ as an effective destabilizer, and NaCl as a representative of the essentially "inert" class of salts (see von Hippel and Schleich, 1969a,b). As a serendipitous result we showed that under some conditions hexanamide and probably pentanamide can be induced to form micelles. This is evidenced by an abrupt change in solubility of these amides with temperature and $NaClO_4$ concentration (Tables I and II and Figures 1 and 3), and by sharp transitions from salting-out to salting-in (Figures 4a, 5a, and 8). Some aspects of these findings are exploited in the following article (Hamabata *et al.*, 1973).

Our solubility measurements, expressed as values of K_s (Table III), show that the average $\Delta K_{s,CH_2}$ (difference be-

TABLE IV: Thermodynamic Parameters for the Transfer of Fatty Acid Amides from Water to 1 M Salt Solutions.[a]

Salt	T (°C)	Hexanamide $\Delta G_{tr,i}$	Hexanamide $\Delta H_{tr,i}$	Hexanamide $\Delta S_{tr,i}$	Pentanamide $\Delta G_{tr,i}$	Pentanamide $\Delta H_{tr,i}$	Pentanamide $\Delta S_{tr,i}$	Butyramide $\Delta G_{tr,i}$	Butyramide $\Delta H_{tr,i}$	Butyramide $\Delta S_{tr,i}$	$\Delta G_{tr,CH_2,av}$	$\Delta G_{tr,Am,av}$
	6	+0.12		+4	+0.05		+4	0		+4	+0.06	−0.18
$NaClO_4$	16	+0.07	+1.1	+4	+0.01	+1.0	+4	−0.05	+1.2	+4	+0.06	−0.23
	25	+0.05		+4	−0.02		+4	−0.08		+4	+0.06	−0.26
	6	+0.30		−2	+0.21		−1	+0.11		−1	+0.10	−0.19
NaCl	16	+0.32	−0.3	−2	+0.22	−0.1	−1	+0.12	−0.2	−1	+0.10	−0.18
	25	+0.33		−2	+0.23		−1	+0.13		−1	−0.10	−0.17

[a] Thermodynamic parameters are calculated as outlined in the text. All $\Delta G_{tr,i}$ and $\Delta H_{tr,i}$ values are in kcal/mol, and all $\Delta S_{tr,i}$ values in entropy units (cal/(mol deg)).

tween the K_s values for two amide-containing compounds differing by one methylene group in the alkyl "tail") are approximately constant for a given salt.

Additional insight into molecular mechanism may be obtained by calculating thermodynamic parameters from the solubility data. Thus for the process of transfer of one mole of a given amide (i) from water to 1 M salt solution at the same concentration we may calculate free energies of transfer from the K_s values given in Table III, since

$$\Delta G_{tr,i} = 2.3RTK_sC_s \quad (4)$$

Values of $\Delta H_{tr,i}$ are derived from the slopes of the best straight lines fitted through plots of K_s *vs.* T^{-1} (°K^{-1}), and these $\Delta H_{tr,i}$ values (assumed independent of T over this range) are combined with the calculated values of $\Delta G_{tr,i}$ to determine $\Delta S_{tr,i}$ for each salt and temperature. Values of $\Delta G_{tr,i}$, $\Delta H_{tr,i}$, and $\Delta S_{tr,i}$ obtained by these means for the three amides are listed in Table IV.

We may see, as expected from the K_s values and the effects of these salts on the stability of biological macromolecules, that the values of the change in free energy of transfer for all three amides to 1 M $NaClO_4$ are lower than those for transfer to 1 M NaCl. The magnitudes of $\Delta G_{tr,i}$ also increase (become more positive) with increasing alkyl chain length and thus increasing polar–nonpolar ratio. We may see that $\Delta G_{tr,i}$ for transfer to $NaClO_4$ decreases with increasing temperature, while this parameter displays a slight tendency in the opposite direction for 1 M NaCl over the temperature range tested.

Table IV also shows that $\Delta H_{tr,i}$ and $\Delta S_{tr,i}$ are positive and approximately independent of temperature for the transfer of all three amides from water to 1 M $NaClO_4$, while these parameters are smaller and slightly negative for transfer to 1 M NaCl.

In the next-to-last column of Table IV, by difference between the earlier columns, we calculate the free energy of transfer from water to $NaClO_4$ or NaCl of an average (nontransitional) methylene group. We obtain ~+60 cal/mol of CH_2 for transfer to 1 M $NaClO_4$ and ~+100 cal/mol of CH_2 for transfer to 1 M NaCl. (Similar values were obtained by Nandi and Robinson (1972b) for $\Delta G_{tr,CH}$ from water to 1 M NaCl by the difference between amino acid side chains differing in length by one CH_2 group.) These values appear to be independent of temperature, which is consistent with the many observations demonstrating that the *change* in T_m per mole of destabilizing salt is essentially independent of the absolute value of T_m for a large variety of biological macromolecules (*e.g.*, see von Hippel and Wong, 1964).

Finally, in the last column of Table IV we calculate the average free energy of transfer of the amide moiety from water to 1 M $NaClO_4$ and 1 M NaCl, respectively ($\Delta G_{tr,Am,av}$). These values are obtained by subtracting five times the relevant value of $\Delta_{tr,CH_2,av}$ from the ΔG_{tr} data for hexanamide, four times $\Delta G_{tr,CH_2,av}$ from the pentanamide data, and three times $\Delta G_{tr,CH_2,av}$ from butyramide values (*i.e.*, we arbitrarily and doubtless partially incorrectly assign a full methylene value to the transitional methylene group for each amide). The average values obtained are quite similar for the two salts, as expected for the demonstrated nonspecific binding of these salts to the "ideal" amide dipole (Hamabata and von Hippel, 1973). The average $\Delta G_{tr,Am}$ calculated for NaCl at 25° is also close to the value of −170 ± 50 cal/mol obtained from the amide group salting-out constant estimated for NaCl at 25° by Schrier and Schrier (1967), and that of ~−150 cal/mol estimated from the data of Nandi and Robinson (1972a,b) for the same solvent and temperature (and using the same group additivity assumptions for CH_2 moieties vicinal to the amide dipole).

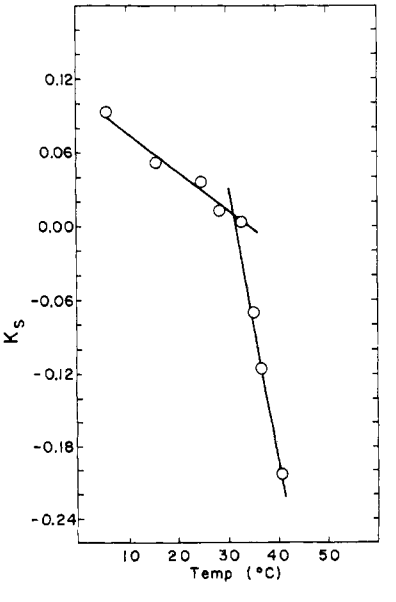

FIGURE 8: Salting-out constants, K_s, for *n*-hexanamide in $NaClO_4$ as a function of temperature.

We may draw some quantitative conclusions from these data with respect to the effects of neutral salts on the stability of biological macromolecules. Taking the average value of $\Delta G_{tr,Am}$ from the last column of Table IV, we may estimate that the free energy of transfer of a peptide group from the interior of protein to a 1 M mono-monovalent salt solution is ~200 cal/mol more favorable than the transfer of the same group to water. The transfer of a methylene group to 1 M NaCl is ~100 cal/mol more unfavorable than transfer of this moiety to water. Since NaCl is "inert" as a conformational perturbant (*i.e.*, it neither stabilizes nor destabilizes the native conformation of biological macromolecules), we may estimate that the average "interior" residue in a protein may be represented by a peptide group and approximately two methylene units. This is in good accord with our previous conclusion (von Hippel *et al.*, 1973) that the acrylamide moiety may be used to represent the average interior group of a protein. On this basis transfer to 1 M $NaClO_4$ destabilizes the folded conformation of a protein by ~80 cal/mol of amino acid residues exposed in the transition and, to the extent that K_s is independent of salt concentration, by, *e.g.*, ~0.5 kcal/mol of residues exposed to 6 M $NaClO_4$. In future studies we plan to test these predictions for macromolecules for which the details of thermally induced unfolding transitions are known or can be surmised.

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Model Studies on the Effects of Neutral Salts on the Conformational Stability of Biological Macromolecules. IV. Properties of Fatty Acid Amide Micelles†

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ABSTRACT: In this article, the anomalous alterations in the rate of change of the solubility of *n*-hexanamide with temperature in water and aqueous salt solutions which were observed previously (Hamabata, A., Chang, S., and von Hippel, P. H., (1973), *Biochemistry 12, 1271*) are shown to be due to the onset of micelle formation in this system. Relative changes in the fluorescent intensity of the dye 8-anilino-1-naphthalenesulfonic acid (ANS), which shows greatly increased fluorescent quantum yields on transfer from an aqueous to a nonpolar environment, are used to monitor micelle formation and to establish critical micelle concentration (cmc) values. The fact that micellar aggregates of fairly discrete size are being established at the cmc is confirmed by preliminary sedimentation velocity experiments. Cmc values are measured fluorimetrically for hexanamide micelles as a function of the concentration of various neutral salts, and it is known that all obey the empirical relation: $\log (\text{cmc}) = \log (\text{cmc})_0 - k_{s,\text{cmc}} C_s$. The parameter k_s varies with salt type, decreasing in approximately the usual Hofmeister order: $NaCl \simeq NaBr > NaI > NaClO_4$. It is shown that $k_{s,\text{cmc}}$ is equivalent to the salting-out coefficients (K_s) obtained from solubility data in preceding articles, and free energies of transfer from water to 1 M salt solutions are calculated from the $k_{s,\text{cmc}}$ data. These values correspond to the net effect of the transfer of that portion of the hexanamide monomer which changes microenvironments in going from the free monomer to the micellar state, and the data obtained are compared with free energies of transfer measured on the same groups in macroscopic systems. The results are used to interpret micelle structure and to illustrate some of the ambiguities which accompany the notions of "interior," "exterior," and "surface" on the macromolecular size scale.

In the preceding article (Hamabata *et al.*, 1973) we reported studies on the effects of various neutral salts on the solubility of fatty acid amides of varying chain length in aqueous solutions. We observed that the solubilities of these amides

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