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Model Studies on the Effects of Neutral Salts on the Conformational Stability of Biological Macromolecules. II. Effects of Vicinal Hydrophobic Groups on the Specificity of Binding of Ions to Amide Groups†

Alberto Hamabata‡ and Peter H. von Hippel*

ABSTRACT: A series of measurements of the affinity of several neutral salts (NaCl, NaBr, NaI, and $NaClO_4$) for water, relative to their affinity for variously substituted small molecule amides, is reported. The measurements were made by binding the salts, as ion pairs, to ion-retardation resins from which the salts can be displaced by water and other polar, but uncharged, eluents. The relative affinity of water and the amides for the salts, as well as the specificity of salt binding to variously substituted amide dipoles, has been determined. It is shown that the affinity of the amides for salt decreases as increasing numbers of methyl substituents are placed around the amide dipole in the order: formamide > acetamide $\simeq$ *N*-methylformamide > *N*-methylacetamide $\simeq$ *N*,*N*-dimethylformamide > *N*,*N*-dimethylacetamide. Thus, the affinity of the amide dipole for neutral salts decreases approximately in proportion to the degree of methyl substitution. However, it is also shown that methyls substituted at different sites have quantitatively different effects on ion binding to the amide dipole. We find that the mono-monovalent salts tested all bind approximately equally to an "ideal" amide dipole resembling, but not identical with, formamide, and that the measured affinities diverge for the various salts as methyl groups are added. The affinity decreases most rapidly per added methyl group for NaCl, and progressively less precipitously for NaBr, NaI, and $NaClO_4$, in that order. Thus, the Hofmeister specificity of neutral salt binding to amides appears to originate in the "modulation" of the binding by the vicinal nonpolar groups. The quantitative results are compared with thermodynamic data obtained by others by solubility measurements, and possible molecular origins of the observed effects are discussed.

In the preceding paper (von Hippel *et al.*, 1973) it was shown that neutral salts bind to the amide groups of polyacrylamide gels with relative (to water) binding constants ($K_{a,rel}$) that follow the rankings of the Hofmeister series in terms of the effects of these ions on the conformational stability of biological macromolecules. In direct proportion to the effect on macromolecular order $\rightleftarrows$ disorder transition temperatures, destabilizing ions (*e.g.*, ClO_4^-, I^-, SCN^-, Ca^{2+}) show positive values of $K_{a,rel}$ for binding to polyacrylamide and "inert" ions (*e.g.*, Cl^-, Na^+, K^+) exhibit relative binding constants close to zero, while ions which tend to stabilize macromolecular conformations (*e.g.*, SO_4^{2-}, F^-) are characterized by negative values of $K_{a,rel}$. Thus, both in terms of the order and the sign of the relative binding constants, the ion-bonding properties of the acrylamide moiety serve as a good model for the behavior of a peptide group and the associated average side chain exposed to solvent as a consequence of protein unfolding or denaturation.

That these measured values of $K_{a,rel}$ represent "real" (relative) binding constants rather than nonspecific activity coefficient effects (*i.e.*, positive or negative ion-exclusion effects due to differences in water structure around the nonpolar groups of the resin) was demonstrated by showing that neutral salts exhibit no preferential binding (positive or negative) to a totally nonpolar polystyrene matrix, relative to a tritiated water tracer. Thus the actual binding site is the amide dipole, and, as shown in the preceding paper, ion effects on the stability of macromolecular conformations can be calculated in terms of the relevant values of $K_{a,rel}$.

However, these results leave unresolved the sources of the specificity of the ion–amide interactions manifested in the Hofmeister series. One possibility that is the specificity is built into the interaction of the ions with the amide dipole itself, regardless of the attached vicinal groups. The other possibility (which is an extension of the proposal of Schrier and Schrier (1967) based on solubility data) is that ion binding to the amide dipole itself is nonspecific for ions of a given charge type, but becomes specific as a consequence of "modulation" of the binding induced by the vicinal hydrophobic groups.

† From the Institute of Molecular Biology and the Department of Chemistry, University of Oregon, Eugene, Oregon 97403. *Received September 27, 1972.* This research was supported by U. S. Public Health Service Research Grant No. GM-15792 (to P. H. v. H.) and U. S. Public Health Service Fogarty International Postdoctoral Research Fellowship No. FO5TW1603 (to A. H.).

‡ Present address: Department of Biochemistry, Instituto Politecnico Nacional, Mexico City, Mexico.

In order to test these competing views directly, we have measured the relative affinities for various ions of amide groups with methyl substituents systematically placed on the amide nitrogen and the carbonyl carbon. These data appear to support the latter hypothesis, in that binding to an "idealized" amide dipole (approximately formamide) is relatively nonspecific for a number of mono-monovalent salts, and specificity of binding develops rapidly as methyl groups are substituted for hydrogens on the amide group. However, contrary to the simple model of Schrier and Schrier (1967), the effects of methyl groups directly adjacent to the amide moiety are not all identical nor are they the same as those due to equivalent groups further removed from the dipole. Rather, the exact effects depend on the immediate chemical environment of each nonpolar group.

In this paper we "map" the effects on ion-binding specificity of methyl groups directly attached to the amide, as well as measuring directly the deviations from simple "functional group additivity" in the resulting amide–ion interactions as a consequence of the presence of methyl groups so placed. In the next paper (Hamabata *et al.*, 1973) it is shown by solubility studies of fatty acid amides that functional group additivity is reestablished for methyl and methylene groups not directly attached to the amide dipole.

The measurements necessary for this study could not be made by the recycling chromatographic procedures employed previously, since appropriately modified column packing materials were not available (see von Hippel *et al.*, 1973). Nevertheless, the small magnitudes of the binding constants expected required the continued use of the amplification inherent in chromatographic procedures. Therefore we decided to invert the previous procedure and use the various amides as the mobile (or eluent) phase, and bind the ions to the stationary phase (column matrix). For these reasons we turned to ion-exchange resins. Pure cation or anion exchange resins could not be used, since ion–ion interactions are much stronger than ion–dipole interactions and thus ions can only be displaced from such resins by other ions, and not by uncharged dipolar molecules such as amides or water. However, ions are primarily bound as ion pairs to the paired acidic and basic sites in mixed cation–anion exchange resins (ion-retardation resins). This makes it possible to elute ions from these resins by ion–dipole interactions (*i.e.*, using an eluting phase containing only water or amide groups). Thus comparison of the relative (to water) elution volumes required to remove various ion pairs from such resins using solutions of substituted amides as eluents provides, with a minimum of assumptions, a measure of the relative binding constants of these amides to the ions involved. Such measurements are described in this paper, and the results are analyzed in terms of the effects of various methyl substituents on the specificity of ion binding to amides.

Materials and Methods

Salts and Amides. All salts used in these studies were reagent grade and were used without further purification. $NaClO_4$ solutions were clarified by passage through 0.45-μ Millipore filters. The amides were obtained as follows: formamide and *N,N*-dimethylformamide from Mallinckrodt; *N*-methylformamide from Aldrich; acetamide and *N,N*-dimethylacetamide from Matheson, Coleman and Bell; and *N*-methylacetamide from Eastman. The amides were generally used without further purification, since in control experiments it was shown that redistilled formamide and *N,N*-dimethylformamide showed identical binding behavior with that of the undistilled materials.

Column Materials. The ion-exchange resin used was AG-11A8, 50–100 mesh, lot no. 2903, and was obtained from Bio-Rad. This material is termed an ion-retardation resin, because it binds ions primarily as ion pairs to adjacent (paired) carboxylate and quaternary ammonium sites attached to a rigid polystyrene lattice cross-linked by divinylbenzene groups. This pairing of ion-binding loci permits the elution of bound salts with water, since the fixed groups can self-pair in the absence of ions in the mobile phase. In addition to the paired ion-retardation sites (~1.0 mequiv/ml of bed volume), a few groups (~0.05–0.1 mequiv/ml) occur in unpaired configurations. These sites behave like conventional cation or anion exchange loci, in that ions cannot be eluted from them by water alone (see Bio-Rad Technical Bulletin No. 113, 1963, and below).

The resin shrinks slightly (less than 2%) in the presence of salt. There were no changes in resin dimensions on the passage of amide-containing solvents through the column.

Chromatographic Procedures. The resin was hydrated by stirring in 1 M NaCl for 24 hr, and "fine" particles were removed by decantation. Glass columns (1.1 × 60 cm) were obtained from Ace, and were packed and run at room temperature at a total bed volume of 46 ± 1 ml. Eluents were pumped with an LKB Recychrom variable-speed peristalic pump at a flow rate of ~30 ml/hr. The effluent was collected in 2-ml fractions using a Gilson fraction collector run in either the drop counting or the timed mode.

Prior to each run, the columns were washed thoroughly with 1 M salt, and then equilibrated with 0.005 M solutions of the salt to be chromatographed (see below). Samples of salts, usually 3 ml at 1 M concentration and containing 0.25 μCi of tritiated water (obtained from New England Nuclear) as an internal marker, were applied to the column and then eluted with either an aqueous 0.005 M solution of the same salt as the sample, or with 1 or 2 M solutions of the various amides to be tested for relative elution effectiveness. The aqueous amide solutions also contained 0.005 M concentrations of the "background" salt.

As indicated above, the performance of AG-11A8 as an ion-retardation resin depends on the spatial relationships between pairs of exchange groups of opposite charge. Two adjacent groups serve as an ion-pair binding site, and can self-neutralize in the absence of mobile ions, thus permitting elution of ions from such paired sites with un-ionized solvents. Ions bound to ion-exchange groups which are too distant from groups of opposite charge cannot be eluted with water. Other sites may be partially paired, leading to intermediate situations in which the bound ions can be eluted with water, but with more difficulty, emerging as a broad shoulder behind the main "ion-retarded" peak.

Such partially paired sites also bind ions much more tightly than the "ion-retardation" sites, leading to peak trailing or total adsorption since at an estimated concentration of 0.1 mequiv/ml of such sites, a 46-ml column can bind up to 4.6 mequiv of salt to these loci, thus effectively adsorbing the entire 3.0-mequiv salt sample used in most of the experiments. The difficulties posed by these unpaired or partially paired ion-exchange groups were resolved by using a constant 0.005 M background of the salt under test in all loading and eluting solutions. This background concentration effectively saturates all the "anomalous" sites, so that the test sample interacts only with the non-tight-binding, paired ion-exchange sites.

TABLE I: Relative Affinity of Various Anions for Ion-Exchange Resins.

Salt	Resin: Dowex 2 K^a	Resin: AG-11A8 $E_w{}^b$
$NaClO_4$	32	20.8
NaI	8–13	11.0
NaBr	3.4	3.6
NaCl	1.0	1.0
NaF	0.1	−0.87

[a] The relative affinity of anions for Dowex 2 is defined as: $K = (A^-)_R(Cl^-)_S/(A^-)_S(Cl^-)_R$ where the subscripts R and S refer to anion concentrations in the resin and solution phases, respectively. Data from Peterson (1954). [b] Elution constants from this work; see text for experimental details. The V_{THO} values used in these calculations were: $NaClO_4$, 30.0 ± 1.0 ml; NaI, 30.2 ± 0.8 ml; NaBr, 32.2 ± 0.7 ml; NaCl, 32.4 ± 0.6 ml; NaF, 32.4 ± 0.5 ml. (The data have been normalized to unity for NaCl; the actual value of E_w for NaCl was 0.433.) Each number represents up to 15 runs, and the values given show an average standard error of ~±2%.

Ion–amide binding constants (relative to ion–water binding constants) were determined (see Results) by measuring the elution volume of a particular salt with water, and comparing this value with the elution volume shown by the same salt eluted with water containing various concentrations of amide. Experiments were run in pairs on the same column, an amide run immediately following the control run with water as an eluent. No variation in results was observed in runs in which the amide elution experiment preceded the water control.

To ensure that the measured elution volumes represent equilibrium data, flow rates ranging from 3 to 60 ml/hr were tested. In all cases the positions of the salt, tritiated water (THO), and amide peaks were found to be independent of pumping speed.

Experiments were run under conditions in which the fixed, paired, ion-retardation sites were always in local excess relative to the concentration of the salt sample. This was demonstrated by showing that the elution volume of the salt peak was independent of salt concentration over the range 0.2–1.0 M, and of sample volume between 1 and 3 ml.

Nonspecific adsorption of amides to the resin was ruled out by showing that the elution volumes of several amide samples (3-ml samples of formamide at 1–8 M concentrations and 1 M *N*-methylformamide or *N*,*N*-dimethylacetamide) were very close to the elution volume for tritiated water (V_{THO}). V_{THO} itself is very constant, falling between 30 and 31 ml in the presence of various amides, at ~30 ml in the presence of NaI and $NaClO_4$, and at ~32 ml in NaCl and NaBr.

Aqueous formamide solutions at moderately high concentrations are somewhat unstable, hydrolyzing spontaneously to formic acid and ammonia to the extent of ~0.01%/hr. The effect of the introduction of formate at these levels was tested by carrying out control runs with 0.005 M sodium formate. No effects on peak positions of formamide, THO, NaCl, or $NaClO_4$ were seen, provided that the usual "background" salt concentrations were used. Nevertheless, freshly prepared amide solutions were used in all runs to avoid any possible artifacts due to amide degradation.

Assays. The salt peaks were assayed using a Radiometer CDM2 conductivity meter equipped with a CDC 114 flow-through cell (dead volume ~ 0.15 ml) mounted at the bottom of the column. Peaks were recorded on a modified PRR recorder (Texas Instruments) attached to the conductivity meter. Salts were also assayed using specific ion electrodes (Orion), and THO was determined by liquid scintillation counting, as described previously (von Hippel *et al.*, 1973). Amide peak positions were determined spectrophotometrically at 230 nm.

Results

The effects of progressive increases in the molarity of small molecule amide on the volume of aqueous eluent needed to elute a particular salt peak from the "paired" sites on an AG-11A8 ion exchange column can be used to calculate binding constants of the ions to each amide, relative to the binding constants for the same ions to water. To this end we may define an elution constant, E_w, for a given salt eluted with water as

$$E_w = \frac{V_s{}^w - V_{THO}}{V_{THO}} \quad (1)$$

where $V_s{}^w$ is the elution volume for a given salt eluted with water (containing "background" levels of the same salt to saturate the unpaired sites on the column—see Materials and Methods) and V_{THO} is the elution volume for a tritiated water marker run simultaneously (*i.e.*, on the same column under the same solvent conditions).[1] E_w is therefore a dimensionless volume ratio which is independent of column size and provides a measure of the affinity for the column of the salt under test, relative to its affinity for water.

In Table I we summarize values of E_w obtained for the various salts tested with the AG-11A8 columns run under the conditions described in Materials and Methods. We may note that the relative affinities of the various sodium salts for the column cover a 20- to 30-fold range. These values are also compared in Table I with the relative affinities of the same series of sodium salts for the quaternary ammonium (anion) exchange resin, Dowex 2, as measured by Peterson (1954). Clearly the relative affinities are very similar for the two types of resins, which is reasonable since the resin matrices and the anion exchange group are the same in both types of columns, though elution is by ion displacement in one case and by water (followed by the self-neutralization of bound paired groups) in the other. Thus, for both types of columns these results may be taken to reflect the relative affinity of the various anions for quaternary ammonium groups attached to a polystyrene matrix. (See Robinson and Jencks, 1965, for further comments on the Dowex 2 data.) In subsequent calculations of the relative affinity of the bound ions for water and

[1] V_{THO} is not an ideal front marker, since (see von Hippel *et al.*, 1973) it is retarded somewhat relative to the "real" front by exchange with water hydrogens immobilized on the column. However, as discussed in Materials and Methods, V_{THO} is essentially constant for all salts tested and in particular is not altered by the presence of amides (in concentrations up to the highest molarity tested) in the eluent. Thus THO provides a satisfactory normalization marker for relating elution constants for water to those obtained with amide-containing eluents.

TABLE II: Relative Distribution Coefficients (Water–Amide Binding Ratios) for Various Salts on a Series of Simple Amides.[a]

Amide	[Amide] (M)	$D_{w/A}$ NaCl	NaBr	NaI	NaClO
Formamide	1	0.952	0.988	0.955	0.940
	2	0.864	0.900		
N-Methylformamide	1	1.125	1.013	1.000	0.980
	2	1.148	1.091		
N,N-Dimethylformamide	1	1.320	1.175	1.131	1.055
	2	1.664	1.344		
Acetamide	1	1.127	1.080	1.071	1.000
N-Methylacetamide	1	1.342	1.187	1.140	1.090
	2	1.743	1.368		
N,N-Dimethylacetamide	1	1.576	1.191	1.125	1.075
	2		1.344		

[a] Values of $D_{w/A}$ averaged from at least three runs for each point. For all sets of runs the standard error in $D_{w/A}$ was less than ±0.01.

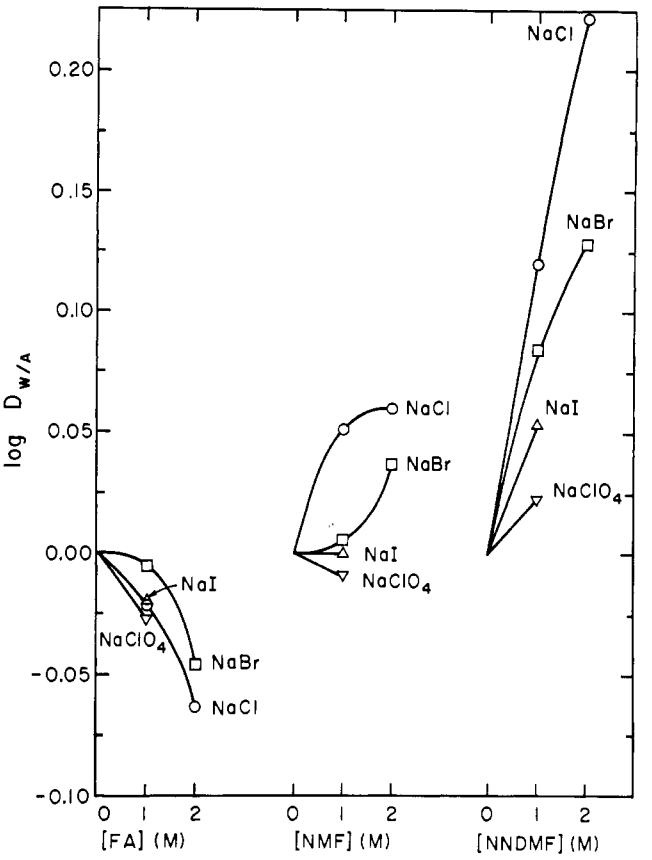

FIGURE 1: Plots of log $D_{w/A}$ *vs.* molarity of amide in the eluent for the effects of various sodium salts on the formamide series of model compounds: ○, NaCl; □, NaBr, △, NaI; ▽, $NaClO_4$; FA, formamide; NMF, *N*-methylformamide; NNDMF, *N,N*-dimethylformamide.

amides the actual values of E_w cancel. However, they are of considerable practical significance, since they indicate that the elution of an $NaClO_4$ peak requires about 20-fold as much time and solvent as the elution of an NaCl peak.

Distribution constants characterizing the affinity of the various salts for amide-containing eluent *relative* to water as the eluent may be defined as follows

$$D_{w/A} = \frac{E_{amide(M_i)}}{E_w} = \frac{V_s^{amide(M_i)} - V_{THO}}{V_s^{w} - V_{THO}} \qquad (2)$$

where $D_{w/A}$ is the relative distribution constant, $E_{amide(M_i)}$ represents the elution constant for water containing amide at molarity M_i (plus "background" salt), and $V_s^{amide(M_i)}$ represents the elution volume for the amide-containing solvent with amide molarity M_i. By this definition $D_{w/A}$ falls below unity when the affinity of the salt for the amide exceeds that for water, and above unity when the converse is true. In principle, $D_{w/A}$ values could be extrapolated to pure amide eluents, but in practice we have held the amide concentrations to values less than 2 M in order to avoid the discontinuous changes in solvent structure, column properties, etc., which might accompany the transition to largely nonaqueous solvents.

In Table II and Figures 1 and 2 we summarize measured values of $D_{w/A}$ obtained with two series of related simple amides, which vary with respect to the number and location of methyl groups around the amide moiety. (Figures 1 and 2 are plotted as log $D_{w/A}$ *vs.* amide concentration for reasons described in the Discussion.) In these summaries the formamide series includes the parent compound, *N*-methylformamide, and *N,N*-dimethylformamide. The acetamide series continues with *N*-methylacetamide and *N,N*-dimethylacetamide.

Perusal of Table II and Figure 1 shows that the differences in the affinity of the various neutral salts tested for the amides of the formamide series become progressively more pronounced as methyl groups are added. Binding to the formamide series is fairly nonspecific, though the differences which remain follow approximately the order characteristic of the Hofmeister series (*i.e.*, ClO_4^- shows a smaller value of $D_{w/A}$ than Cl^-, indicating that ClO_4^- binds to formamide somewhat more tightly, etc.). Also, for formamide the value of $D_{w/A}$ shows a markedly nonlinear dependence on amide concentration, and $D_{w/A}$ falls below unity for all the salts tested. As we move to *N*-methylformamide, the differences in $D_{w/A}$ for the various salts become more pronounced, and the $D_{w/A}$ values rise above unity for Cl^- and Br^-. For *N,N*-dimethylformamide, the differences between the various salts become striking, and all the values of $D_{w/A}$ now fall above unity.

Table II and Figure 2 summarize the behavior of $D_{w/A}$ as a function of amide concentration for the acetamide series. For this series log $D_{w/A}$ is approximately a linear function of amide concentration for all the amides, and the Hofmeister order is obviously manifested from the start. Again the differences between the values of $D_{w/A}$ for different salts become more pronounced (the plots "fan" out) as the first *N*-methyl group is added to acetamide (to form *N*-methylamide). The addition of a second *N*-methyl group to *N*-methylacetamide (to make *N,N*-dimethylacetamide) has virtually no additional effect on the magnitudes of the dependence of $D_{w/A}$ for any of the salts (except NaCl) on amide concentration. This is in strong contrast to the effect of the addition of the second *N*-methyl group in the formamide series, where it significantly spreads and linearizes the fan-shaped plots.

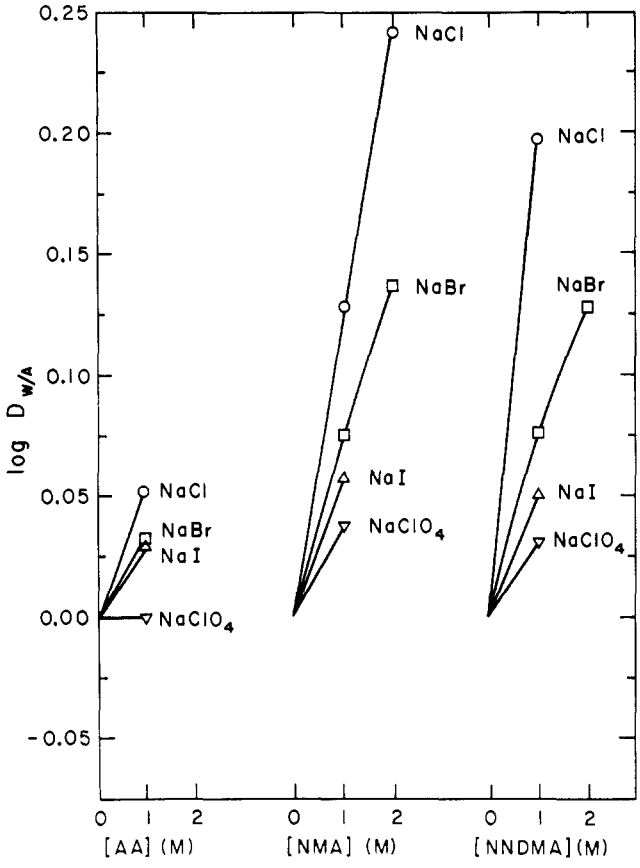

FIGURE 2: Plots of log $D_{w/A}$ *vs.* molarity of amide in the eluent for the effects of various sodium salts on the acetamide series of model compounds: ⊙, NaCl; ⊡, NaBr; △, NaI; ▽, $NaClO_4$; AA, acetamide; NMA, *N*-methylacetamide; NNDMA, *N,N*-dimethylacetamide.

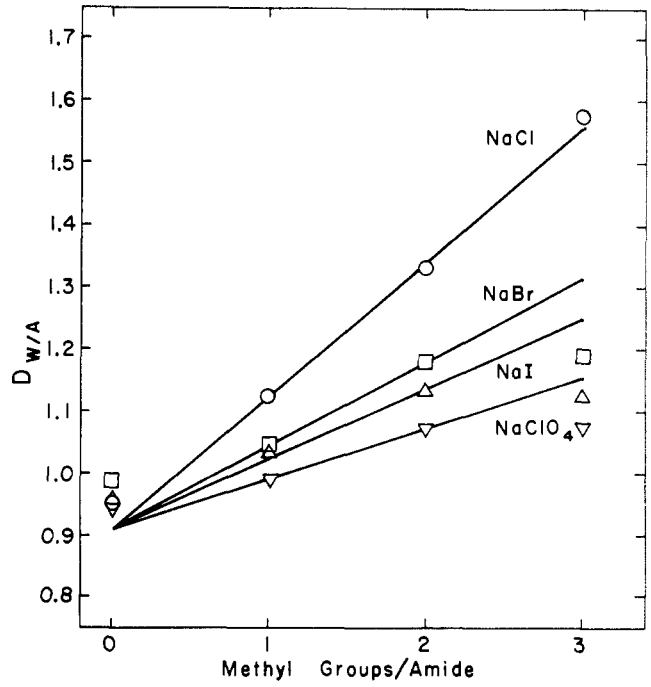

FIGURE 3: Plot of $D_{w/A}$ *vs.* the number of methyl groups attached to the amide dipole, for various salts and eluent solutions containing 1 M amides. The points at "zero" methyl groups correspond to formamide, those at one methyl to the average of *N*-methylformamide and acetamide, those at two methyls to the average of *N*-methylacetamide and *N,N*-dimethylformamide, and those at three methyls to *N,N*-dimethylacetamide: ⊙, NaCl, ⊡, NaBr; △, NaI; ▽, $NaClO_4$.

Discussion

Effect of Methyl Group Location. In the preceding article (von Hippel *et al.*, 1973) it was demonstrated that neutral salts bind to amide groups attached to a nonpolar matrix with a relative affinity which follows the Hofmeister series. In this article we have shown that the *differences* between the relative affinity of amide groups for a series of mono-monovalent salts depend on the number and distribution of methyl groups around the amide dipole, with the differences between the salts increasing (*i.e.*, the Hofmeister specificity developing) as the number of vicinal methyl groups is increased. A semiquantitative view of this phenomenon is presented in Figure 3, where we plot the $D_{w/A}$ data for the formamide and acetamide series (for the 1 M amide eluents) as a function of the number of methyl substituents on the amide dipole. The points for one and two methyl groups represent, respectively, the average of the *N*-methylformamide and the acetamide data, and the average of the *N*-methylacetamide and *N,N*-dimethylformamide data. The zero methyl group points, of course, refer to formamide, and the three methyl group points to *N,N*-dimethylacetamide. The data show clearly that the lines for different salts diverge (fan-out) with increasing number of methyl groups. Figure 3 also shows that a family of straight lines drawn through the average one and two CH_3 groups data extrapolate closely to a common point on the ordinate (zero methyl groups), and that this point falls at a $D_{w/A}$ value of ~0.91, somewhat below the cluster of points for formamide itself. We interpret this result to indicate that binding to an "ideal" amide dipole is indeed essentially nonspecific, as suggested by Schrier and Schrier (1967), and that formamide itself falls somewhat short of "ideality," *i.e.*, it retains some residual Hofmeister specificity. Nandi and Robinson (1972a) have also recently shown that neutral salts demonstrate some vestigal Hofmeister-type specificity on the solubility of formamide. We may speculate that formamide binds ions somewhat less strongly (*i.e.*, is salted-out somewhat more—see below) than the "ideal" amide dipole as a consequence of the presence of the various hydrogens which are replaced by methyl groups in the higher members of the series. These hydrogens could confer some residual hydrophobic character on the formamide molecule. This speculation is, at least, consistent with the experimental demonstration that the activity coefficient of molecular hydrogen (H_2) in aqueous salt solutions varies with salt type in accord with the predictions of the Hofmeister series (see Long and McDevit, 1952).

The convergence of the extrapolated data (plotted either linearly as in Figure 3, or logarithmically) at approximately zero methyl groups appears to confirm the hypothesis that binding to the amide dipole itself is nonspecific for salts of a particular charge type. This conclusion was reached by Schrier and Schrier (1967) (and extended to the results of Robinson and Jencks (1965) on acetyltetraglycyl ethyl ester) by assuming that all methyl (or methylene) groups have equivalent effects on amide solubility (or, in our terms, amide binding) in a particular salt, regardless of the location of the methyl group. The results obtained in this study show this assumption to be quantitatively incorrect, at least for some of the methyl groups attached directly to the amide dipole. Thus, while a linear increase in $D_{w/A}$ is observed for all the

salts tested in going from the ideal amide dipole to the average one and then two methylamide compounds, this progression breaks down almost completely for *N,N*-dimethylacetamide (Figure 3). Other deviations are apparent within the formamide (Table II and Figure 1) and the acetamide (Table II and Figure 2) series.

Specifically, a comparison between $D_{w/A}$ values for *N*-methylformamide and acetamide (or *N*-methylacetamide and *N,N*-dimethylformamide) suggests that a methyl group attached to the amide carbon has a marginally larger "negative modulating" effect (*i.e.*, is more effective in decreasing salt binding to the amide dipole) than has a methyl attached to the amide nitrogen. A second methyl group attached to the amide nitrogen has essentially no additional effect on salt binding to the amide dipole of the acetamide series (except for NaCl) while the second *N*-methyl does further reduce ion binding to the formamide series of compounds.

Comparison with Thermodynamic Data Obtained by Solubility Measurements. We next attempt to correlate quantitatively the $D_{w/A}$ values reported in this paper with classical salting-out data derived from solubility measurements. It has been shown by many workers (*e.g.*, Long and McDevit, 1952; Robinson and Jencks, 1965; Nandi and Robinson, 1972a,b) that the effects of salt concentrations on the activity coefficients (relative solubilities) of nonelectrolytes generally follow the Setschenow equation

$$\log f_i = K_s C_s \tag{3}$$

where f_i is the activity coefficient of the nonelectrolyte in question (usually defined as S_0/S_s where S_0 is the solubility of the nonelectrolyte in water and S_s its solubility in salt concentration, C_s), and K_s is the salting-out constant characteristic for each salt. K_s is usually defined as the slope of a plot of f_i *vs.* C_s, or the initial slope of such a plot if the data are not linear over the entire salt concentration range tested. Negative values of K_s correspond to a net salting-in effect, and positive values to net salting-out (*i.e.*, a decrease in solubility with increasing salt concentration). In these solubility experiments the thermodynamic data refer to the process of transferring a mole of amide from an infinite volume of water to an infinite volume of aqueous salt solution of salt concentration C_s.

$D_{w/A}$ has been defined as the ratio of elution constants for water and for various aqueous amide solutions. Therefore here the elementary process involves, in effect, the transfer of a mole of *salt* from an infinite volume of water to an infinite volume of *amide* solution of amide concentration C_{Am}.[2] By analogy with eq 3, we may write for this process

$$\log D_{w/A} = K_{Am} C_{Am} \tag{4}$$

where K_{Am} is the initial slope of a plot of log $D_{w/A}$ *vs.* C_{Am}, and

$$\Delta G_{tr,s} = RT \ln D_{w/A} = 2.3RTK_{Am}C_{Am} \tag{5}$$

where $\Delta G_{tr,s}$ represents the free energy of transfer of a mole of a particular salt, s, from water to a particular amide solution of concentration C_{Am}. By this convention, negative values of K_{Am} (or $\Delta G_{tr,s}$) correspond to preferential binding of the salt to the amide (values of $D_{w/A}$ less than unity). To the extent that the water component stays constant we can treat the solution as a two-component system of salt and amide to which a two-component Gibbs–Duhem equation should apply; *i.e.*, we may write

$$\left(\frac{\partial \Delta G_{tr,s}}{\partial n_{Am}}\right)_{n_{H_2O}} = K_{Am} = \left(\frac{\partial \Delta G_{tr,Am}}{\partial n_s}\right)_{n_{H_2O}} = K_s \tag{6}$$

where *n* corresponds to the mole fraction of the component indicated by the subscript and therefore K_{Am} must equal K_s, at least for infinitely dilute solutions of amide and salt, respectively. In Table III we compare K_s values for various salts and amides taken from the work of Nandi and Robinson (1972a) and Schrier and Schrier (1967) with values of K_{Am} from these studies. (K_{Am} is defined by the 1 M amide points, since no data were taken at lower amide concentrations.) Considering the very different ways in which the data were obtained the agreement is remarkably good, and clearly supports the interpretation we have taken of the thermodynamic basis of our measurements. (See also note added in proof.)

TABLE III: Comparison of Amide–Ion Interaction Parameters Obtained from Relative Eluent Volumes and from Solubility Data.

Amide	Salt	K_{Am} (M^{-1})[a]	K_s (M^{-1})
Formamide	NaCl	−0.02	−0.04[b]
	NaBr	−0.005	−0.03[b]
	NaI	−0.02	−0.04[b]
	$NaClO_4$	−0.03	−0.08[b]
Acetamide	NaCl	+0.052	+0.05[b]
	NaBr	+0.033	+0.03[b]
	NaI	+0.030	
	$NaClO_4$	0.000	0.00[b]
N-Methylacetamide	NaCl	+0.128	+0.11,[b] +0.099[c]
	NaBr	+0.074	+0.08,[b] +0.065[c]
	NaI	+0.057	+0.018[c]
	$NaClO_4$	+0.037	+0.03[b]

[a] The 1 M amide data are used in this table. See text for definition of K_{Am} and justification for comparing it with the salting parameter, K_s. [b] From Nandi and Robinson (1972a). [c] From Schrier and Schrier (1967).

We may also note that the zero methyl intersection point of our data (Figure 3) leads to a K_{Am} value for the ideal amide dipole of ~ -0.04 M^{-1}. This may be compared with the average K_s value of -0.1 ± 0.03 M^{-1} obtained for the interaction of mono-monovalent salt with the amide group by Schrier and Schrier (1967), assuming functional group additivity and estimating the ratio of the salting-out constants of the methyl and methylene groups on the basis of specific volume ratios. It is also in reasonable accord with the estimate of $K_s = -0.13$ M^{-1} made by the same authors for a simple ion–dipole interaction between a mono-monovalent salt and the amide dipole using a modified Debye–McAuley equation.

Molecular Mechanisms. We have the following facts at our disposal in attempting specific molecular interpretations of the results presented here. In the preceding article (von Hippel *et al.*, 1973) it was shown that no significant (preferential to

[2] Actually, this is only strictly true if we neglect the small changes in V_{THO} which accompany the changes in amide and salt type and concentration (see Table I). For our measurements this assumption is well justified, since the measured changes in V_s (eq 1) greatly exceed those in V_{THO}.

water) ion binding (positive or negative) occurs to the nonpolar groups of the polystyrene matrix, but that the Hofmeister specificity of ion binding is fully developed for binding to the acrylamide moiety of polyacrylamide. In this paper it is shown that binding is nonspecific to the ideal amide dipole, and that therefore the specificity of binding to polyacrylamide must arise as a consequence of the insertion of vicinal methyl groups on the acrylamide group. These methyls, which do not participate directly in binding ions, must therefore influence directly the effectiveness and specificity with which the ions bind to the amide dipole. And finally, it has been shown that methyl groups far removed from the amide dipole (Nandi and Robinson, 1972b; Hamabata *et al.*, 1973) or isolated nonpolar groups (Long and McDevit, 1952) are salted-out of aqueous solution by ions with the characteristic Hofmeister specificity.

Two general types of mechanisms can be conceived for the vicinal methyl group effects on ion binding: either these groups have an inductive effect on the amide dipole which introduces a specificity of ion binding to the amide, or the nonpolar groups exert their effects by interacting with the "water structure" in their immediate vicinity, thus perturbing the solvent structure associated with the amide–salt complexes. The "inductive effect" hypothesis seems unlikely, largely because methyl substituents in the various positions have such different effects (Figures 1 and 2).

We can view the "water structure modification" mechanism in several related ways. We may speculate, following the Frank and Evans (1945) and Kauzmann (1959) approach to hydrophobic bonding, that methyl groups induce partial clathrate water structures about themselves, and that these water structures then interfere with the binding of ions to neighboring groups. The polystyrene column results reported in the preceding article require that such a water structure be fully accessible to ions. Alternatively we may argue that the nonpolar groups fit rather well into unperturbed water structure, but are hydrated less effectively (*i.e.*, salted-out) by the differently organized water surrounding ions. In either view we can reason that it is the differences in the ability of the various ions to reorganize water structure (which we interpret as the molecular basis of the Hofmeister series) that account for the methyl group effects on relative ion binding to the amide dipole. Thus, specifically ClO_4^- and I^-, which are more effective water structure disorganizing anions than Br^- and Cl^- (see von Hippel and Schleich, 1969a,b, for detailed summaries of the evidence on this point), show much smaller decreases in affinity for the amide dipole as a consequence of the presence of the vicinal methyl groups than do the Br^-- and Cl^--containing salts (Figure 3).

Of course, these problems can also be considered explicitly from the point of view of the ions. For example, in a very recent paper, Taylor and Kuntz (1972) have argued that the mechanistic basis for the Hofmeister series of anions may lie primarily in the varying abilities of these ions to act as proton acceptors in hydrogen bonding with the solvent. Regardless of one's initial approach, all interpretations appear to involve competitive water reorganization: by the ions, by the nonpolar groups, and by the amides; and the net effect on the various species must reflect the outcome of this multicomponent competition. Thus it is obvious that a final understanding of these problems still awaits a detailed unravelling of the equilibrium structures of water in the presence of various ions, dipoles, and nonpolar groups, and an appreciation of the thermodynamic consequences of these structures. (See note added in proof.)

In any case, with respect to the vicinal methyl groups, there is considerable precedent for suggesting that the extent to which water structure is perturbed by such groups depends upon the nature of the atoms in the molecule to which the nonpolar group is directly attached. For example, von Hippel and Wong (1965) pointed out in introducing the concept of "effective methylene groups" in denaturing solvent additives that the first methylene group of each hydrocarbon arm of the tetraalkylammonium series is rendered ineffective as a contributor to the net denaturing effectiveness of the total molecule by proximity to the presumed "water structure neutralizing" influence of the central charged nitrogen atom. A similar argument was made for the effect on methylene groups of vicinal hydroxyl groups in difunctional alcohols (*e.g.*, ethylene glycol). Thus in the present context we can argue that a less extensive water structure might be organized around a methyl group attached to the partially positively charged nitrogen of the amide dipole than around a methyl attached to the carbonyl carbon, or alternatively that a methyl group attached to such a partially charged nitrogen might have less difficulty in accommodating itself to the water rearrangements resulting from the presence of a nearby ion.

In conclusion, then, we attribute the total effect of ions on the solubility of a partially polar (amide group containing), and partially nonpolar, molecule to the sum of a nonspecific ion-binding effect to the polar part, and a specific salting-out effect based on competitive water reorganization around the nonpolar parts. Nonpolar groups adjacent to the polar amide group contribute to both components, the water structure effects associated with them contributing (and providing specificity) to the ion–amide binding interaction as well as to the water structure based salting-out effect. The exact location of the "transition" methyl groups will determine the extent to which water organization about them is affected, and thus the magnitudes of their contributions to the above binding and salting-out phenomena. In the following article (Hamabata *et al.*, 1973), it will be shown that the effects of additional methylene groups further removed from the amide dipole are additive and show the full Hofmeister series ion specificity of salting-out effects.

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Added in Proof

This thermodynamic analysis involves dilute solutions of amide or salt, respectively, and the extrapolation of the concentration of the other component involved to zero. Thus it is reasonable to treat the chemical potential of the water component as constant in these systems. On the other hand, in the polystyrene (and polyacrylamide) experiments reported in the preceding paper (von Hippel *et al.*, 1973), *none* of the components of the system can be assumed to show constant chemical potential. Some molecular insight can be obtained by a thermodynamic consideration of these systems.

Following Klotz (1966) we may write a three-component Gibbs–Duhem equation for these systems

$$n_{H_2O}d\mu_{H_2O} + n_s d\mu_s + n_{np}d\mu_{np} = 0$$

where μ_i and n_i are the chemical potential and mole fraction of component i, and the subscripts H_2O, s, and np refer to the water, salt, and nonpolar solute components, respectively.

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†

Alberto Hamabata,‡ Susan Chang, and Peter H. von Hippel*

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

† From the Institute of Molecular Biology and the Department of Chemistry, University of Oregon, Eugene, Oregon 97403. *Received September 27, 1972.* This research was supported by Research Grant No. GM-15792 (to P. H. v. H.) and U. S. Public Health Service Fogarty International Postdoctoral Research Fellowship No. FO5TW1603 (to A. H.).

‡ Present address: Department of Biochemistry, Instituto Politecnico National, Mexico City, Mexico.