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Ion Effects on the Solution Structure of Biological Macromolecules

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Biological macromolecules, and particularly the proteins and nucleic acids with which we shall be primarily concerned in this Account, consist basically of one or more linear copolymer chains. These chains contain about twenty (proteins) or about four (nucleic acids) chemically distinct types of monomer units, linked together by peptide or sugar phosphate bonds in a genetically determined sequence, and range from 10^2 to 10^6 residue units in length. Thus within the limits of monomer sequence and backbone stereochemistry, any particular chain or set of interacting chains is potentially capable of assuming an enormous number of three-dimensional conformations.

Yet in aqueous solution at moderate temperature and pH, a given set of polypeptide or polynucleotide chains is generally found to be disposed in space in one or a small number of closely related structures. Furthermore these are almost certainly equilibrium structures, since it has been shown with several systems that macromolecules can be reduced to random coil polymers and then "reannealed" into the original conformation by judicious manipulation of the solvent environment. It is thus generally believed that the particular "native" structure of a protein or nucleic acid represents a state of minimum free energy, and furthermore that the free energy of this state is the algebraic sum of the free energies of the individual residue–residue and residue–solvent contacts involved in the equilibrium conformation.

In marked contrast to the structural properties of the usual small molecule, the native conformation of most proteins and nucleic acids is only marginally stable, and often a decrease of only a few hundred calories per mole in one of the stabilizing interactions involved in maintaining this form is enough to trigger a cooperative transition to a quite different structure. This borderline structural stability can easily be rationalized since the existence of specific conformations requires a delicate balance of intermolecular interactions. Thus some preferential residue–residue interactions are required to give the macromolecule an "inside" and a specific three-dimensional structure (fully preferential residue–solvent interaction leads, of course, to a random coil conformation), while some favorable residue–solvent contacts are required to maintain a stable "outside" of the macromolecule and to prevent uncontrolled intermolecular interaction resulting in precipitation or phase separation.

This marginal conformational stability, together with the cooperative character of macromolecular transconformation reactions, is also important in functional terms since it permits the easy interconversions from one form to another which may often accompany the activity of these molecules as enzymes, genetic templates, and so forth. However it represents a major headache to those physical biochemists who attempt to predict macromolecular conformations by summing up the free energies of the intra- and interchain interactions involved in various structures, or who attempt to evaluate the contributions of individual types of residue–residue or residue–solvent interactions to overall structural stability.

Nevertheless in this Account we will describe some aspects of the structural stability of biological macromolecules and consider how this stability might be altered by interaction of various components of the macromolecule with a variety of structural perturbants (particularly electrolytes) which can be added to the solvent environment.

Classification of Perturbants

Most work on the effects of perturbants on macromolecular structure has been done with proteins or protein models, and the first widely recognized perturbants used to destabilize ("denature") native protein structures were agents with obvious hydrogen-bonding potential, such as urea and guanidinium chloride. Since it had long been thought that proteins are largely held in their specific secondary–tertiary–quaternary structure by hydrogen bonds, and since several molar solutions of urea and guanidinium chloride had been found to bring about the conversion of compact globular proteins to disordered random coil forms, it was assumed that these agents worked by competing

more successfully than water for the donors and acceptors involved in intramolecular hydrogen bonding in the native protein.[1] This view, while probably not entirely incorrect, must be considerably modified by the finding that these same agents increase appreciably the solubility of alkanes and nonpolar amino acids,[2] which clearly shows that competitive hydrogen bonding is not the whole story. Also the development of the concept of hydrophobic bonding as a major determinant of protein stability[3] has tended to undermine the older, simplistic view. Finally, and most obviously in nucleic acids, charge–charge interactions can also be shown to play a major role in determining the stability of macromolecular conformations.

Thus, to a first approximation, we can attempt to classify perturbants as potential hydrogen-bond, hydrophobic-bond,[4] and electrostatic-interaction affectors. However, it is immediately apparent that very few structural perturbants can be classified as "pure" affectors of one of the above types of interactions, and it is further apparent that ionic species can potentially effect all three of these types of interactions.

Specifically, then, we shall adopt the structural and operational concept of a biological macromolecule implied above (which follows directly from the basic ideas developed by Kauzmann[3] and Tanford[5] and their collaborators, and many others), *i.e.*, that a macromolecule consists of residues which are so disposed as to form an "inside," consisting of residues which are not in contact with solvent, and an "outside," consisting of residues which are in contact with solvent, and the major functional expression of perturbants which increase or decrease the stability with respect to conversion to a fully solvated random coil is to increase or decrease the free energy of transfer of residues from the inside of the protein into the solvent environment.

This thermodynamic effect can obviously have its origin in either of two general types of mechanisms (or a combination of both). (1) Small molecule perturbants can bind directly to the various interior groups of the folded macromolecule (which is in dynamic equilibrium with various unfolded forms so that its interior residues are continuously, though transiently, exposed to the solvent), thus increasing or decreasing the solubility of these structural elements in the external surround. (2) Perturbants can modify the organization of solvent around potentially exposed groups, thus increasing or decreasing the free energy of transfer of these groups to the exterior through effects on local solvent structure. Since most biological macromolecules seem only to attain the biologically active native conformation in water or largely aqueous solutions, we infer that the properties of water and its potentials for interaction with the various functional groups of macromolecules must play a decisive role in any final explication of conformational stability.

Macromolecular Manifestations

Often the simplest way to induce a reversible folding $\rightleftarrows$ unfolding or order $\rightleftarrows$ disorder transformation in a protein or nucleic acid is to increase the temperature of the solution by 10 to 50°. If this is done gradually and the effect monitored by some physiochemical criterion sensitive to conformation, such as viscosity, ultraviolet hypo- or hyperchromism, optical activity, etc., it is seen that unfolding occurs quite abruptly over a rather narrow temperature range. By analogy with the not dissimilar behavior of macroscopic phase transitions, the temperature of the midpoint of this unfolding range is called the melting temperature (T_m). Thus an experimentally simple way to measure the *net* effect of adding a small molecule perturbant to the solvent environment is to determine the change in T_m with changing additive concentration.

This type of effect is illustrated in Figure 1 for the thermally induced conversion of collagen from the triple-helical ordered form to the disordered random coil conformation in the presence of various concentrations of $CaCl_2$.[6] As this figure implies, neither the structure of the ordered nor of the disordered form of collagen is changed appreciably by the addition of $CaCl_2$, but T_m (the temperature at which the helical and the coil forms are equally stable, *i.e.*, at which $\Delta G = 0$ and therefore $T_m = \Delta H/\Delta S$ for a two-state transition) is moved to progressively lower temperatures with increasing concentrations of $CaCl_2$. Furthermore, the shift in T_m is approximately linear with molarity of $CaCl_2$ (T_m decreases $\sim$16°/mol of $CaCl_2$ added), and the effect is nonsaturating, at least at concentrations up to 1 M.

Such experiments are generally carried out at macromolecule concentrations of $\sim$1 mg/ml. Thus, for proteins, the residue concentration is generally less than 10^{-2} M. Therefore if we write the reaction scheme for the unfolding of the macromolecule in the presence of a particular perturbant as eq 1 and 2, where eq 1 repre-

$$r_{interior} \rightleftarrows r_{exterior} \quad (1)$$

$$r_{exterior} + C \rightleftarrows r_{exterior}C \quad (2)$$

sents the transfer of an interior residue into the solvent environment and eq 2 represents the binding of a single molecule of perturbant (C) to each residue, and if the observed effects are due entirely to preferential binding to the newly exposed residues, then the association constant for eq 2 must be of the order of 0.01 M^{-1} (see below).

Plots similar to that shown in Figure 1 could have been presented to document the similar destabilizing

(1) A. E. Mirsky and L. Pauling, *Proc. Natl. Acad. Sci. U. S.*, **22**, 439 (1936).

(2) Y. Nozaki and C. Tanford, *J. Biol. Chem.*, **238**, 4074 (1963); D. B. Wetlaufer, S. K. Malik, L. Stollen, and R. L. Coffin, *J. Am. Chem. Soc.*, **86**, 508 (1964).

(3) W. Kauzmann, *Advan. Protein Chem.*, **14**, 1 (1959).

(4) For present purposes we shall operationally define a hydrophobic bond affector as one which *increases* the solubility (*decreases* the activity coefficient) of a hydrocarbon-like model compound relative to the solubility of that compound in pure water.

(5) C. Tanford, *J. Am. Chem. Soc.*, **84**, 4240 (1962).

(6) P. H. von Hippel and K.-Y. Wong, *Biochemistry*, **2**, 1387 (1963).

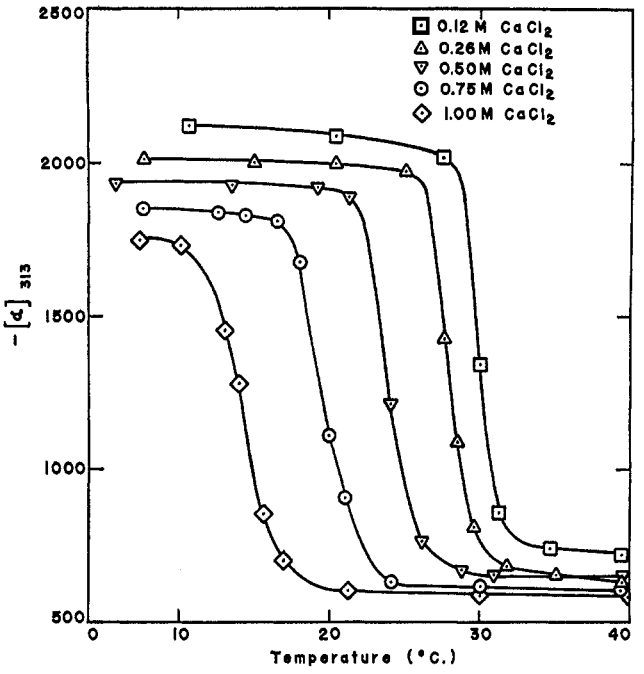


Figure 1. Melting curves for ichthyocol collagen in various concentrations of $CaCl_2$, pH 7, ~1 mg of collagen/ml and: □, 0.12 *M* $CaCl_2$; △, 0.26 *M* $CaCl_2$; ▽, 0.50 *M* $CaCl_2$; ○, 0.75 *M* $CaCl_2$; ◇, 1.0 *M* $CaCl_2$ (from ref 6).

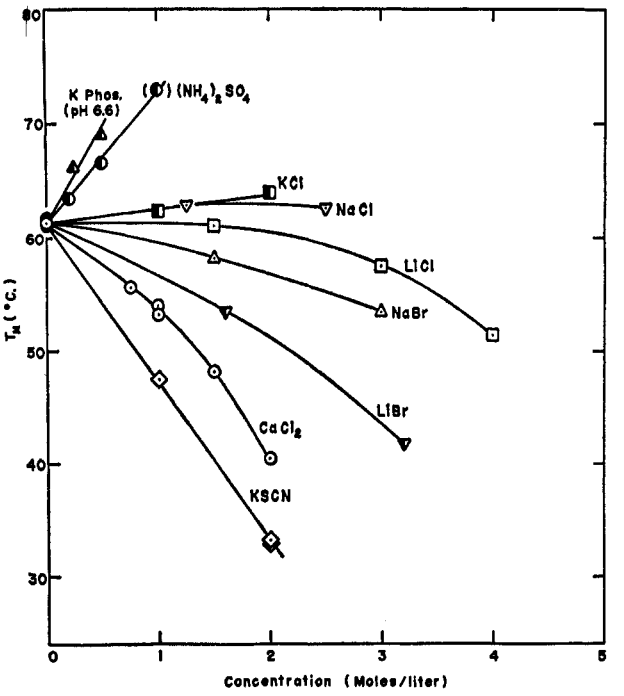


Figure 2. Transition temperatures of ribonuclease as a function of concentration of various added salts. All of the solutions were adjusted to pH 7.0 and also contained 0.15 *M* KCl and 0.013 *M* sodium cacodylate; ribonuclease concentration, ~5 mg/ml (from ref 8).

effect of $CaCl_2$ on the temperature-induced folding ⇄ unfolding transitions of macromolecules as diverse as globular proteins (ribonuclease), α-helical fibrous proteins (myosin), and nucleic acids (DNA), suggesting that the effects of this salt on the stability of the ordered form transcend chemical and conformational details of macromolecular structure.[7]

The effects on the thermal transition temperature of ribonuclease of a number of neutral salts at various concentrations are shown in Figure 2.[8] This figure shows that while a given salt may have a similar effect on the stability of a variety of macromolecules, the magnitude and the direction of the effect differ widely from one salt to another. Thus while some salts (KSCN, $CaCl_2$) are potent destabilizers of the ordered structure, others (KH_2PO_4, $(NH_4)_2SO_4$) are potent stabilizers of the ordered form, and still others (KCl, NaCl) are quite inert as either stabilizers or destabilizers. In general, these plots of T_m *vs.* C_s (salt concentration) are relatively linear, especially at higher salt concentrations, and so the effect of any particular salt on macromolecular stability can be described by an approximate empirical equation of the form

$$T_m - T_m^0 = K_m C_s \qquad (3)$$

where T_m^0 and T_m are the melting temperatures of the macromolecule in pure water and in the aqueous perturbant solution of concentration C_s, respectively, and K_m is the slope of a plot of T_m *vs.* C_s. Thus K_m may be thought of as a measure of the molar effectiveness of a given salt as a perturber of macromolecular stability, where a negative value characterizes a destabilizing salt, and a positive value a stabilizing salt.[9]

This generality of the effects of ions on the stability of macromolecular structures transcends intramolecular folding ⇄ unfolding reactions and, as has been documented in detail elsewhere,[10] applies to all systems in which there is a net transfer of residues from an unsolvated to a solvated environment. Thus, as Figure 3 shows, ions which effectively stabilize macromolecular conformations, *i.e.*, which increase $\Delta G_{transfer}$ for residues from the inside of the macromolecule to the outside, also are effective salting-out agents for macromolecules; *i.e.*, they increase the chemical potential of the exterior groups of the macromolecule, thus favoring the formation of a separate insoluble phase. Similar effects can be seen on protein subunit association ⇄ dissociation equilibria, on macroscopic phase transitions (melting of polymers, shortening of tendons), on enzyme activities (the maintenance of the active site is, of course, depen-

(7) P. H. von Hippel and K.-Y. Wong, *Science*, **145**, 577 (1964).
(8) P. H. von Hippel and K.-Y. Wong, *J. Biol. Chem.*, **240**, 3909 (1965).

(9) These are obviously not nonspecific electrostatic effects, since both the magnitude and the direction of the effects depend on the specific ions involved. However, at low salt concentrations macromolecules with large net charge do show a dependence of T_m on salt concentration, which deviates markedly from the simple behavior predicted by eq 3 and may be superimposed upon it. For example, at *low salt concentrations*, the T_m of DNA shows a *logarithmic* dependence on the concentration of monovalent cation, T_m increasing about 18° per tenfold increase in salt concentration. Theoretical calculations have shown that this effect of salt can be almost completely attributed to suppression of the interchain electrostatic repulsion between negatively charged phosphates in native DNA (*e.g.*, see S. Lifson and C. Schildkraut, *Biopolymers*, **3**, 195 (1965)). Comparable effects (though smaller in magnitude) should also be expected in proteins with appreciable net charge.

(10) P. H. von Hippel and T. Schleich in "Biological Macromolecules," Vol. II, S. Timasheff and G. Fasman, Ed., Marcel Dekker, New York, N. Y., 1969, p 417.

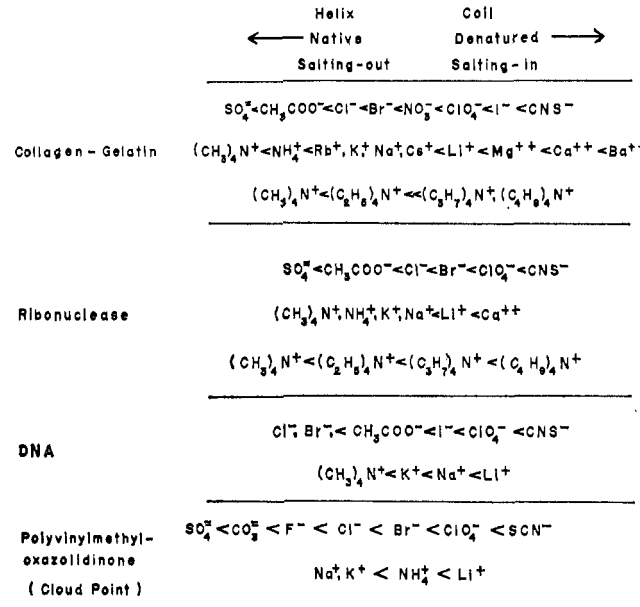


Figure 3. Relative effectiveness of various ions in stabilizing or destabilizing the "native" form of collagen, ribonuclease, DNA, and polyvinylmethyloxazolidinone in aqueous solution. See text for references to the studies from which these rankings were derived.

dent on the over-all native geometry of the macromolecule), and on the kinetics of macromolecular folding $\rightleftarrows$ unfolding reactions.[10]

Figures 2 and 3, by implication, also bring out the following additional facts about the effects of the various ions on macromolecular structure (for details and documentation, see ref 10).

(1) Cations and anions are additively effective in altering T_m (and thus the free energy of transfer; see below). In fact, this additivity has been shown to extend well beyond the simple neutral salt systems. For example, the destabilizing effects of $CaCl_2$ and ethylene glycol on the T_m of the ribonuclease transition sum quantitatively, and the destabilizing effects of the guanidinium salts are greatly increased if the guanidinium cation (Gu^+) is coupled with an anion such as SCN^- or ClO_4^-, rather than with the conventional but relatively inert Cl^-. On the other hand, Gu_2SO_4 is a net stabilizing agent, the T_m-depressing effect of the Gu^+ ion being more than offset by the stabilizing activity of the SO_4^{2-} moiety.[8]

(2) In order of effectiveness as salting-out agents (Figure 3) the anions and cations both follow rather closely the classical series first demonstrated by Hofmeister for the salting-out of euglobulins. This series has also been shown to apply to the salting-out of a number of simple nonpolar nonelectrolytes from solution (for an extensive compilation of such data, see Long and McDevit[11]).

(3) The tetraalkylammonium ions increase in destabilizing effectiveness with increasing length of alkyl side chain. It has been shown that the effectiveness of these cations, *per side chain*, is directly comparable to that of the analogous aliphatic alcohol or acid which is one methylene unit shorter.[8]

(4) Certain polymers (*e.g.*, polyvinylmethyloxazolidinone or poly-L-proline) form a separate phase when heated to a critical temperature (T_c). Salting-out agents tend to lower T_c (stabilize the insoluble phase) while salting-in or macromolecular destabilizing agents raise T_c in accord with the rankings shown in Figure 3.[12]

It is clear that the various ions have specific and predictable effects on the stability of a wide variety of macromolecular structures and complexes. What, then, are the mechanisms by which perturbants bring about these effects?

Free Energy of Transfer of Model Compounds

In several places above we have stated that the effects of the various structural perturbants on the stability of macromolecules in aqueous solution can be viewed as effects on the free energy of transfer of the interior groups of the macromolecule which are exposed to the solvent as a consequence of the unfolding transition. One way to subject this idea to quantitative test is to measure the free energy of transfer of appropriate model compounds from a nonpolar to an aqueous phase containing different concentrations of the various structural perturbants under consideration. Charged groups are not generally found inside macromolecules, presumably because the "thermodynamic cost" of burying such groups in the nonpolar interior is too great. On the other hand, the individual residues which comprise a polypeptide chain are held together by polar peptide bonds, and thus perforce in burying a highly nonpolar sequence of amino acid residues in the macromolecular interior one also buries these polar moieties. Likewise the heterocyclic purine and pyrimidine bases which "stack" upon one another in the native DNA structure, and as a consequence are partially removed from contact with the solvent, also contain polar –NHCO– groups as well as nonpolar and presumably hydrophobic methylene groups. It is, of course, this partially hydrophobic and partially hydrophilic interior of biological macromolecules which gives them a specific structure, and simultaneously makes the native form only marginally stable when compared, for example, to a micelle of sodium dodecyl sulfate, where the interior is completely nonpolar and like a "liquid drop"; *i.e.*, it has no specific internal structure. Thus good models for such free energy of transfer studies include compounds containing both peptide groups and nonpolar components, or purine and pyrimidine rings. Such studies have been carried out by a number of workers,[13] and Figures 4 and 5 show some representative results, obtained with acetyltetraglycyl ethyl ester ($Ac(Gly)_4Et$) and with adenine, respectively.

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

(12) I. M. Klotz, *Fed. Proc.*, **24**, S-24 (1965); A. Ciferri and T. A. Orofino, *J. Phys. Chem.*, **70**, 3277 (1966).

(13) (a) D. R. Robinson and W. P. Jencks, *J. Am. Chem. Soc.*, **87**, 2470 (1965); (b) D. R. Robinson and M. E. Grant, *J. Biol. Chem.*, **241**, 4030 (1966); (c) E. E. Schrier and E. B. Schrier, *J. Phys. Chem.*, **71**, 1851 (1967).

These studies differ slightly from the transfer experiments described above in that, for technical reasons, these workers have chosen to study the transfer of the model compound from a solid phase to the aqueous solution. Thus what is actually being measured is the change in the solubility of the model compound in the aqueous solution containing different concentrations of various neutral salts, and this change in solubility has been defined in terms of the activity coefficient of the model compound in the aqueous phase. The activity coefficient, γ, is defined by eq 4, where S_0 = the solubility of the model compound in pure water and S = the solubility in aqueous solution containing perturbant. (γ_0, the activity coefficient for the model compound in pure water, is defined as unity in this formulation.) It is well known that the salting-out of nonelectrolytes can often be represented by the empirical Setschenow equation,[11] which we may write

$$\gamma = S_0/S \tag{4}$$

$$\log \gamma = \log (S_0/S) = K_s C_s \tag{5}$$

where K_s is the salting-out coefficient for a given salt and C_s is salt molarity. By analogy with the empirical equation which applies to macromolecular stability (eq 3), effective salting-out agents are characterized by positive values of K_s, and salting-in agents by negative K_s values.

Figures 4 and 5 show that the salting-out (or -in) of $Ac(Gly)_4Et$ and adenine follow the Setschenow equation quite well, and furthermore, with some exceptions, that the relative effectiveness of various ions as salting-in or -out agents is very comparable to the Hofmeister series which applies to macromolecules (Figure 3). However, there are significant quantitative differences; thus salts like NaCl, which had essentially no effect on macromolecular stability ($K_m \simeq 0$), are significantly effective salting-out agents for $Ac(Gly)_4Et$ and adenine ($K_s > 0$), while agents like LiCl and NaBr, which have appreciable destabilizing potency ($K_m < 0$), are essentially ineffective as perturbers of the solubility of the model compounds ($K_s \simeq 0$). Some clue as to the significance of these differences may be had by studying the salting-out behavior of benzene (Figure 6). Here we see that *all* the salts tested, including NaI and $NaClO_4$, act as salting-out agents for this totally nonpolar com-

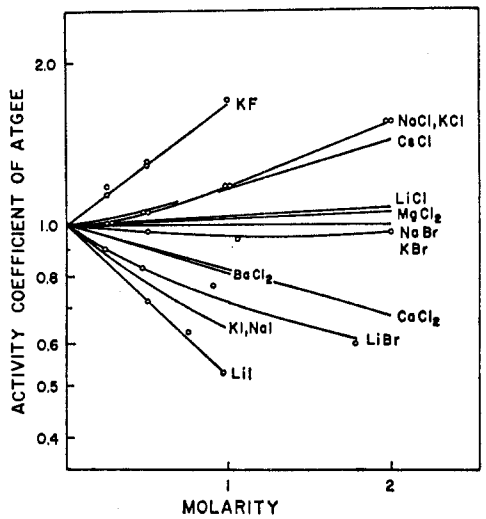


Figure 4. Activity coefficients of acetyltetraglycyl ethyl ester $Ac(Gly)_4Et$ in solutions of various salts at 25.0° (from ref 13a).

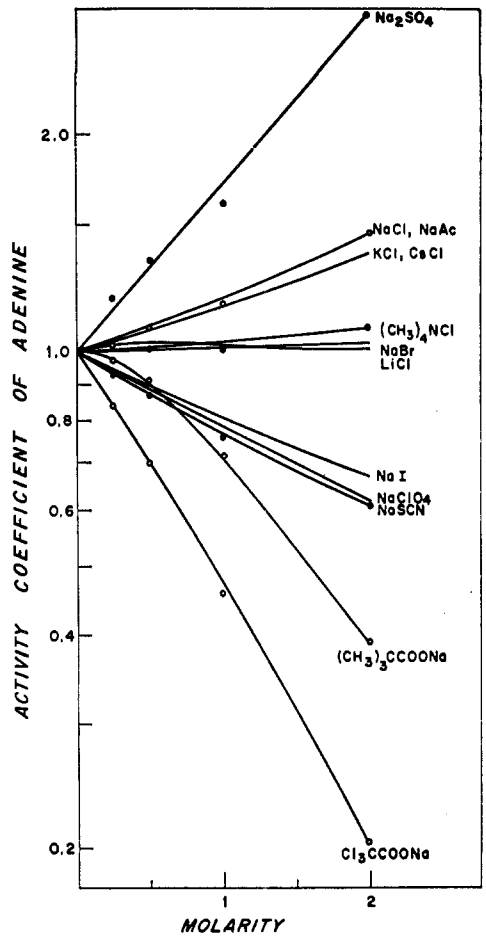


Figure 5. Activity coefficients of adenine in aqueous salt solutions at 25° (from ref 13b).

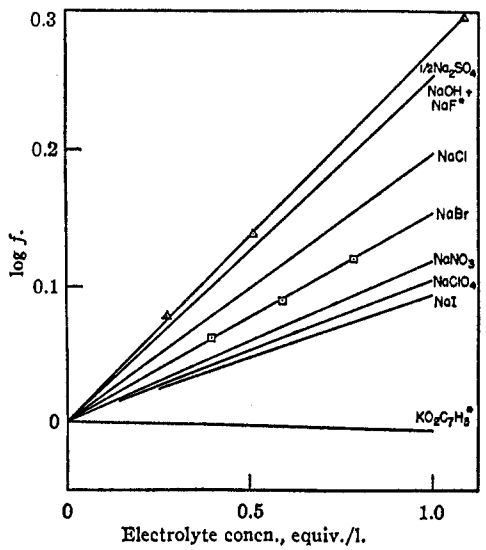


Figure 6. Effects of sodium salts on activity coefficients of benzene, 25° (from ref 11).

Table I

Salting-out Parameters for Aqueous Amides at 25°

Salt	$K_{s,NMP}$[a]	$K_{s,NMA}$[a]	$K_{s,Ac(Gly)_4Et}$[b]	k_{s,CH_2}[a]	$k_{s,A}$ Ac(Gly)$_4$Et[b]	$k_{s,A}$ NMA–NMP[a]
NaCl	0.164	0.099	0.046	0.065	−0.100	−0.109
NaBr	0.118	0.065	0.00	0.053	−0.083	−0.105
NaSCN	0.025	−0.023	−0.25	0.048	−0.115	−0.177
NaI	0.056	0.018	−0.23	0.038	−0.100	−0.104
LiCl	0.077	0.033	0.021	0.044	−0.078	−0.107
LiBr	0.025	−0.004	−0.17	0.029	−0.090	−0.096
LiSCN	−0.052	−0.082	−0.30	0.030	−0.103	−0.178
$CaCl_2$	0.166	0.076	−0.09	0.090	−0.178	−0.212
$CaBr_2$	0.057	0.000	∼−0.35	0.057	∼−0.185	−0.182
Na_2SO_4	0.486	0.372	0.48	0.114	−0.145	+0.008

[a] Data from ref 13c. [b] Data from ref 13a.

pound, though the relative effectiveness of the various salts still follows the Hofmeister series.

The differences between these various effects have been put on a quantitative basis by Schrier and Schrier,[13] who carried out a comparative study of the salting-out behavior of N-methylacetamide (NMA) and N-methylpropionamide (NMP). They found that all the neutral salts tested were more effective salting-out agents for the slightly more nonpolar N-methylpropionamide than for N-methylacetamide and use the data obtained (plus a few reasonable assumptions) to break up the model compounds into constituent groups, each characterized by a group-specific salting-out constant, as given in eq 6 and 7. They then used the salting-out

$$K_{s,NMA} = 2k_{s,CH_3} + k_{s,A} \quad (6)$$

$$K_{s,NMP} = 2k_{s,CH_3} + k_{s,CH_2} + k_{s,A} \quad (7)$$

coefficients calculated for the methylene (k_{s,CH_2}) and ethyl acetate ($k_{s,EtOAc}$) groups for each salt to calculate an amide group salting-out coefficient ($k_{s,A}$) from the Ac(Gly)$_4$Et data of Robinson and Jencks, as in eq 8.

$$K_{s,Ac(Gly)_4Et} = k_{s,EtOAc} + 4k_{s,CH_2} + 4k_{s,A} \quad (8)$$

Some results are summarized in Table I. We may note that all salts are effective salting-out agents for the nonpolar methylene groups ($k_{s,CH_2} > 0$), but that the relative effectiveness of the various salts as salting-out agents varies as predicted by the Hofmeister series. On the other hand, $k_{s,A}$ seems to be negative for all the salts and is reasonably constant (with some exceptions) for all salts of a particular charge type, such as the monomonovalent salts. Furthermore, the values of $k_{s,A}$ obtained have been shown to be in reasonable accord with what one might calculate for the salting-out of a polar nonelectrolyte using a modified Debye–McAuley equation.[13]

Schrier and Schrier[13] have taken the analogy between the macroscopic K_m and the group salting-out coefficient k_s still further. By combining a relation between the melting temperature of crystalline polymers as a function of diluent concentration[14] with the Setschenow equation, they obtained eq 9, where T_m^0 and T_m are the

$$T_m - T_m^0 = [R(T_m^0)^2/\Delta H_{res}]C_s\Sigma N_i k_i \quad (9)$$

melting temperatures of the macromolecule in pure water (or standard dilute salt) and in the presence of a particular salt at concentration C_s, ΔH_{res} is the enthalpy of melting per residue of the polymer under consideration (assumed independent of temperature), N_i is the mole fraction of polar or nonpolar groups exposed in the transition, and k_i is the relevant salting-out coefficient for the groups in the particular salts involved. Making some not unreasonable assumptions about the type and number of groups exposed in the transition and using the values of k_1 obtained from the free energy of transfer of model compound studies, these workers were able to obtain rather good agreement between calculated values of T_m and experimental melting data on collagen[15] and on ribonuclease.[8,13]

These studies suggest the following broad picture: (1) The effects of salts on the stability of macromolecules can be quantitatively correlated with the effects of these agents on the free energy of transfer of appropriate model compounds from a nonaqueous environment into the aqueous milieu. (2) The specific effects of the various ions arise primarily through their effects on the chemical potential of the nonpolar portions of groups exposed in a macromolecular unfolding or depolymerization reaction, while interaction with the newly exposed amide (or polar) groups provides a constant salting-in bias.[16] (3) Whether a particular salt will serve as a stabilizer or destabilizer of the "native" state (or as a salting-in or salting-out agent for a particular model compound) depends on the ratio of polar to nonpolar groups in the portion of the macromolecule exposed by the unfolding or depolymerization reaction (or by the polar/nonpolar ratio of the model compound under study). Apparently the observation that the same set of salts is characterized by $K_m \simeq 0$ for most macromolecules tested may be attributed to the fact

(14) P. J. Flory, *J. Cellular Comp. Physiol.*, **49**, Suppl. 1, 175 (1957).

(15) P. H. von Hippel and K.-Y. Wong, *Biochemistry*, **1**, 664 (1962).

(16) It should be noted, however, that the *magnitude* of this bias will often depend on a number of factors, such as the dipole moment of the polar portion of the exposed group. Furthermore, specific salt effects in addition to those involving the nonpolar portions may arise if the exposed groups are appreciably acidic or basic.[11]

that the polar/nonpolar ratio of the groups exposed in thermally induced unfolding or depolymerization reactions at moderate temperatures is approximately the same in these macromolecules.

Approaches to Mechanism

While these conclusions are all subject to minor reservations and qualifications, they provide a useful working model of how the stability of macromolecules is controlled by the structure and composition of the solvent environment. However, many unanswered questions remain. (1) How *is* the free energy of transfer of the nonpolar components of macromolecules (*i.e.*, the solubility of benzene) affected by different salts? Are these specific effects on the water structure which develops around the nonpolar moiety itself, or do they depend on direct interaction between perturbant and the nonpolar groups? (2) If the specific, nonpolar salting-out component depends only on effects on water structure, is there a specific thermodynamic or spectroscopic parameter which can be measured on simple ion–water solutions and with which the specific parts of the macromolecular and free energy of transfer effects can be directly and quantitatively correlated? (3) Is the salting-in interaction with the amide groups a binding phenomenon, and is it really constant for all salts of a given charge type, as suggested by the calculations of Schrier and Schrier? (4) If there is binding to the amide dipole, where on the amide group do the anions and cations bind, and what effects do they have on the molecular structure and properties of the amide group?

Some possible approaches to these questions, weighted heavily toward those we happen to be taking in our own laboratory, are outlined in the following discussions.

Binding of Ions to Amide-Containing Model Compounds. *Thermodynamic Approach.* It can easily be shown that *if* the effects on the stability of macromolecular structure or on the free energy of transfer of model compounds are completely or partially due to ion binding, then this binding must be weak ($K_{assoc} \simeq 0.01$–$0.05\ M^{-1}$ for a typical destabilizing salt; see above). Such calculations, using these types of data, have been made by Bello,[17] Mandelkern and Stewart,[17] and Robinson and Jencks.[13] However, there exist in the literature no *direct measurements* of such binding constants, and it is clear from an estimate of the orders of magnitude expected that straightforward approaches, such as equilibrium dialysis with radioactive ions, are not feasible. Some measurements do exist on the binding of ions to charged groups in ion-exchange resins and to the charged groups of proteins, but we are interested in binding to the amide dipole. One approach we have recently employed is to measure the retardation (relative to a tritiated water marker) of various neutral salts on columns of pure polyacrylamide, $[-CH_2CH(CONH_2)-]_n$. The columns were thermostated so that relative retardations could be measured as a function of temperature, and recycling techniques were used to increase the effective column lengths (theoretical plate number) sufficiently to achieve good separation. The detailed findings will be presented elsewhere,[18] but in qualitative outline the following results were obtained: The various ions tested bound to polyacrylamide (relative to the tritiated water marker) as predicted by the Hofmeister series (Figure 3). The binding of any one salt was the sum of anion and cation effects. Good destabilizers (I^-, ClO_4^-, SCN^-, Ca^{2+}) were retarded (positively bound) relative to water. Inert ions (Na^+, K^+, Cl^-) moved essentially with the water peak. Structure stabilizers (SO_4^{2-}, F^-) were *negatively* bound relative to water; *i.e.*, they moved *ahead* of the water peak.

The retardation (binding) constants showed large temperature effects, with the preferential binding (relative to water) of all the strongly retarded (positively bound) ions decreasing markedly with increasing temperature. Preliminary binding constants calculated from these data are comparable to those estimated previously[13,17] from K_m and K_s values.

None of the neutral salts showed binding to uncharged column packing materials devoid of amide groups, including dextran and (except for the long-chain tetraalkylammonium ions) polystyrene.

Thus these results provide a measure of the binding, *relative to water binding,* of these ions to the amide dipole *attached to the hydrophobic polyethylenic backbone of the polyacrylamide polymer.* If taken as a direct measure of the simple ion–dipole binding interaction, these results appear in conflict with expectations based on the work of Schrier and Schrier, who showed $k_{s,A}$ (and thus presumably the amide–ion binding constant) to be essentially invariant for salts of any one charge type. Rather, in terms of the experiments on the free energy of transfer of model compounds cited above, the apparent relative binding constants obtained on the polyacrylamide columns seem directly analogous to the *total* K_s values found. This analogy is further strengthened by the large temperature dependence found for the relative binding constants, which is much greater than that expected for simple ion–dipole interactions and resembles, in magnitude and direction, the temperature dependence of K_s for $Ac(Gly)_4Et$ in the presence of KI.[13]

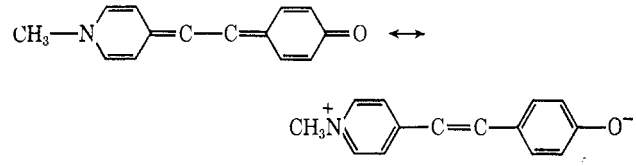
However, perhaps this analogy is not so surprising if we remember that the polar/nonpolar ratio for acrylamide is quite comparable to that of some of the model amides discussed above, as well as that ion binding is being measured *relative* to water binding. Further insight into the factors involved might well be obtained if such ion–dipole binding measurements could be extended or extrapolated to amide groups *not* surrounded by hydrophobic methylene groups (*e.g.*, formamide). Such measurements are currently in progress.

One additional finding which supports the suggestion that ion binding to the amide dipole itself might be

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

(18) P. H. von Hippel, V. Schleich, and L. Schack, in preparation.

quite nonspecific for ions for any one charge type comes from some recent results of Davidson and Jencks[19] on salt effects on the solubility and spectra of the merocyanine dye (I), which is a member of the resonating



I

vinylogous amide class of compounds. Clearly here the two ends of the amide dipole are widely separated, but nevertheless, in the presence of a variety of neutral salts, Davidson and Jencks observed spectral shifts which were saturatable and showed isosbestic points. Assuming these shifts to be due to a spectrally distinct salt–amide complex, these workers calculated apparent association constants for complex formation in aqueous solution and found that, for all the uni-univalent salts tested, $K_{assoc} \simeq 1.0\ M^{-1}$. The various salts also affected the solubility of the merocyanine dye in water, and these effects, unlike the apparent binding constants, followed the classical Hofmeister pattern (Figure 3).

"Mapping" Approach. In addition to thermodynamic measurements of the salt–amide binding constants, it is also of interest to know *where* on the amide the salts bind and what effect (if any) this binding has on the geometry and properties of the peptide (amide) bond. It is well known that the amide group is describable in terms of two resonant states, A and B, and

A B

that this resonance is responsible for the dimensions and planarity of the peptide linkage. The above resonance scheme endows the amide nitrogen with a partial positive charge, and the carbonyl oxygen with a partial negative charge, which would be expected to lead to anion binding at or near the nitrogen and cation binding at or near the oxygen. These expectations are supported by X-ray studies on LiCl–N-methylacetamide complexes and on other crystallized salt–amide systems.[20] The X-ray results have been extended, and further insight into changes in the resonance behavior of the amide dipole at acid pH in the presence of various salts has been obtained in a recent nmr study of the exchange rate of the amide hydrogen of N-methylacetamide in the presence of various neutral salts.[21] The observed effects on the hydrogen exchange rate again do not follow the usual Hofmeister series.

Throughout this discussion it has been asserted that the primary effects of the neutral salts on macromolecular stability result from ion-induced changes in the free energy of transfer of interior residues into the solvent milieu. Thus, it is of some interest to consider the effects of salts on a conformational transition involving little or no change in the net exposure of residues to the solvent environment.

We have recently completed such a study[22] on the salt-induced noncooperative conversion of the extended, semirigid, poly-L-proline II structure to a more random form. The poly-L-proline II structure (Figure 7) is held in its extended geometry because little or no free rotation is possible about the $\geqslant C_\alpha$–CO– bond (the angle ψ is essentially fixed) as a result of steric restrictions, rotation about the >N–$C_\alpha\leqslant$ bond (angle ϕ) is prevented by the pyrrolidine ring, and the angle ω (rotation about the >N–CO– bond) is fixed by the partial double-bond character of the peptide link. We find that the simple inorganic cations all have equal effects on the conformational stability of poly-L-proline II, while the anions vary widely in molar destablizing effectiveness. We have interpreted these data and those of others in terms of ion binding to the amide dipole and have suggested that the anions induce a variable increase in the partial double bond character of the peptide group (favoring form B above), in approximate proportion to the anionic polarizability. This is accompanied by a concomitant lengthening of the $\geqslant C_\alpha$–CO– bond which increases the range available to the angle ψ and thus induces the noncooperative collapse of the poly-L-proline II helix. These stereochemical consequences of ion binding alter the conformation of poly-L-proline II only because of its unique mode of structural stabilization and would not be expected to have such effects on the over-all conformation of nonpyrrolidine-containing macromolecules. Furthermore, as expected, these effects do not follow the usual Hofmeister ion series. The salting-out of poly-L-proline II, which again involves phase equilibria, is also observed under some conditions in these experiments and again *does* follow the Hofmeister order.

Free Energy of Transfer of Nonpolar Compounds. Another experimental approach to an improved understanding of neutral salt effects on macromolecular conformational stability involves measurement of the free energy of transfer of totally nonpolar compounds into aqueous solutions containing the various perturbing salts. Experimental approaches which may be used include: (1) study of salt effects on the solubility of the nonpolar alkanes in aqueous ionic solutions and (2) examination of salt effects on the critical micelle concentration (cmc) of various detergents. In both of these areas considerable, though scattered, experimental information already exists.

The model on which this discussion is based leads us to expect that all salts should "salt-out" the alkanes from aqueous solution, with a relative molar effectiveness which follows the Hofmeister series. The available

(19) S. J. Davidson and W. P. Jencks, *J. Am. Chem. Soc.*, **91**, 225 (1969).

(20) J. Bello, D. Haas, and H. R. Bello, *Biochemistry*, **5**, 2539 (1966).

(21) T. Schleich, R. Gentzler, and P. H. von Hippel, *J. Am. Chem. Soc.*, **90**, 5954 (1968).

(22) T. Schleich and P. H. von Hippel, *Biopolymers*, in press.

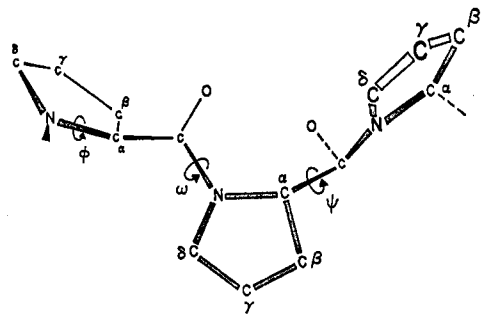
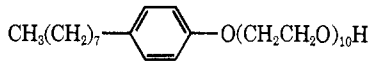


Figure 7. Schematic drawing of poly-L-proline II, showing bond distances and angles cited in the text (modified from P. R. Schimmel and P. J. Flory, *Proc. Natl. Acad. Sci. U. S.*, **58**, 52 1967).

data seem in general to bear out these expectations,[23] though certain ions (*e.g.*, guanidinium and sulfate) often behave anomalously.

Many studies have been reported on the effects of salts on the cmc of charged and uncharged detergents. Unfortunately, many of the data on micelles with charged head groups are difficult to interpret because of the complications involved in properly evaluating the influence of the various salts on intramicellar electrostatic interactions. Detergent molecules with polar but uncharged head groups obviate some of the difficulties associated with the charged detergents. A relevant study of the effects of various guanidinium salts on the cmc of such micelles[24] (of Triton X-100) has shown guanidinium ion to be a strong salting-in agent, with the associated anions showing salting-in or -out effects which follow the Hofmeister ranking. The effects of the guanidinium salts on the cmc of these micelles are strikingly similar to their effect on the T_m of ribonuclease.[7,8] However these results may not be representative of the behavior of uncharged micelles in general since the "hydrophilic" head group of Triton X-100 is large relative to the hydrophobic tail and contains hydrophobic ethylene groups within it (II).



II

Correlations between Ion Effects on Macromolecules and on Water Structure

Since effects of ions on the structure of water around nonpolar groups must play a role in the salting-out of nonpolar compounds from aqueous solution (or put another way, since ions must somehow alter the ways in which water chooses to accommodate nonpolar moieties) considerable time has been spent by workers in this field in attempting to correlate some thermodynamic or spectral parameter reflecting ion–water interactions with the effects of ions on macromolecules in aqueous solution. This subject has been extensively reviewed elsewhere,[10] and it is found that while some parameters fit reasonably well over a limited range of ions, none is fully satisfactory. As one example of such a study, we have attempted[25] to correlate the magnitudes of the ion-induced changes in the nmr chemical shift of water protons with the effects of these salts on the melting temperatures of macromolecules. A good correlation of this sort was initially demonstrated for the interaction of DNA with sodium perchlorate,[26] and while we have confirmed these findings and shown that they apply equally well to the ribonuclease–perchlorate system, the correlation is much less satisfactory when attempts are made to extend it to other salts.

One possible explanation for the fact that no such correlations have succeeded in a general way is that each experimental parameter evaluated contains effects peculiar to the measurement, as well as effects "seen" by the macromolecules. However it seems to us to be also very likely that the problem of finding the lowest free energy condition for the simultaneous accommodation of ions, various water structures, and macromolecules in either a folded or unfolded state is essentially a three-component problem, and that analysis of the interaction of any two components will provide information which is relevant and necessary, but not sufficient, to the solution of the problem.

Much of the research discussed here which derives from our laboratories was carried out in collaboration with Mr. Kwok-Ying Wong, Dr. David Talbot, Mrs. Virginia Schleich, and Mrs. Lise Schack, as described in the detailed papers to which reference is made, and their major contributions are gratefully acknowledged. The research has been primarily supported by U. S. Public Health Service Grants AM-03412, AM-12215, GM-15792, and 5-K3-GM5479 (to P. H. v. H.), as well as by a Helen Hay Whitney postdoctoral fellowship (to T. S.). We are grateful to Drs. John Schellman, William Jencks, and Robert Baldwin for helpful comments on an earlier draft of this paper.

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(24) W. B. Gratzer and G. H. Beaven, *J. Phys. Chem.*, **73**, 2270 (1969).
(25) T. Schleich and P. H. von Hippel, in preparation.
(26) D. E. Gordon, B. Curnutte, Jr., and K. G. Lark, *J. Mol. Biol.*, **13**, 571 (1965).

Additions and Corrections

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Page 265. Formula II should be