THE ROLE OF SOLVENT INTERACTIONS IN PROTEIN CONFORMATION

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I. INTRODUCTION

The molecular events which make up life processes have as their common substrate water, the only naturally occurring inorganic liquid. Since water existed on this planet long before life evolved, the many biochemical and biophysical processes involving complex conformational changes have had to adapt to the eccentric physical properties of water. These properties are well documented[1,2] but the principle of "familiarity breeds contempt" very much applies to the field of scientific study developed in this review.

In a discussion of the possible influence of the aqueous medium on protein conformational stability, it must be kept in mind that other biopolymers (nucleotides and polysaccharides) and their complexes with smaller molecules, e.g., lipoproteins and nucleohistones, are similarly affected by the solvent medium. Even water-soluble synthetic polymers, such as polyvinyl alcohol, polyvinylpyrrolidone, polyethylene oxide,[3] and polyacrylic acid derivatives exhibit, albeit at a more rudimentary level, some of the peculiar specific conformational behavior which is so characteristic of "native" biological macromolecules.[4]

For the purposes of this review we shall, at times, regard the protein as the sum total of the constituent amino acid residues, each of which is able independently to interact with water by virtue of various functional groups, such as peptide bonds, ionizable groups: COO^-, NH_3^+, other polar groups: OH, SH, etc. At other times we shall treat the protein as an "oil droplet" of colloidal dimensions surrounded by several solvation spheres of subtly different properties. Yet again, at other times we regard the protein as a polyelectrolyte which maintains its stability in an aqueous electrolyte environment by virtue of electric double layer interactions. Finally, in discussing the effects of solvent perturbations on protein conformational stability, we shall draw on the available knowledge about the properties of the mixed aqueous solvents themselves and the effects of such solvent media on processes rather simpler than protein conformational changes. It will become apparent that the often complex influences exerted by ions and nonelectrolytes on protein stability are sometimes closely paralleled in simpler physicochemical processes which can

then, with the necessary caution, be studied as analogs for the process involving the protein.

II. EXPERIMENTAL TECHNIQUES

The solvent involvement in the maintenance or disruption of protein conformational integrity can be monitored by an ever increasing variety of experimental methods. It is beyond the scope of this review to describe in detail these physical techniques, and we shall be more concerned with the type of information which different experimental approaches are able to provide. Quite apart from this, many expert reviews exist of the more common methods used to study protein conformational processes.[5]

It is, however, important to emphasize the scope and limitations of various techniques. Thus the information which can be derived from experimental methods can be broadly classified as:

1. Structural
2a. Thermodynamic (energetic)
2b. "Pseudothermodynamic" (by assignment of specific equilibrium constants derived from nonthermodynamic methods)
3. Dynamic and kinetic, both on a macroscopic and microscopic scale.

Structural information is obtained ideally from a direct measurement of distances and angles. Diffraction and scattering techniques are the most powerful, although in favorable cases nuclear magnetic resonance (n.m.r.) methods are capable of providing such information. At a less detailed level, certain conformational states can be characterized by chiroptical methods, e.g., circular dichroism (CD).

Thermodynamics deals with the interconversion of different forms of energy during physical, chemical, or biochemical processes. The most popular thermodynamic function in physical biochemistry is the Gibbs free energy function ΔG. Knowledge of its second and higher temperature and pressure derivatives further allows the specification of processes in terms of enthalpy, entropy, volume, heat capacity, expansibility and compressibility changes, as shown in Figure 1. These properties cannot by themselves provide any information about binding sites, mechanisms, or rates. Truly thermodynamic measurements include colligative properties (freezing point, vapor pressure, osmotic pressure) sorption isotherms, solute partition (e.g., by chromatography), density (i.e., partial specific volume), compressibility, calorimetry – scanning, isothermal, or adiabatic – i.e., enthalpy and heat capacity changes.

The term "pseudothermodynamic" we apply to all those methods, mainly spectroscopic and chiroptical, which monitor relative concentrations of coexisting, distinguishable species in a mixture, from which an equilibrium constant can be derived. This is then readily converted into thermodynamics by the relationship $-\Delta G^{\ominus} = RT$ in K and, if measurements are available at two or more temperatures, the van't Hoff equation is applied:

$$\frac{\partial \ln K}{\partial T} = \frac{\Delta H^{\ominus}_{vH}}{RT^2}.$$

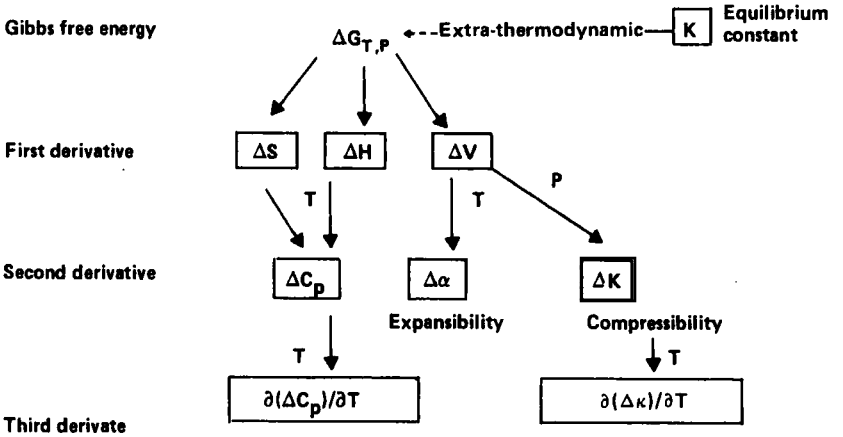


FIGURE 1. Gibbs free energy function and its temperature and pressure derivatives which are directly accessible to experimental determination.

The assumptions implicit in the application and integration of this equation are frequently glossed over, as are also the relationships between ΔH values derived by different methods.[6]

Finally, the dynamic properties of macromolecular and solvent species in a mixture can be derived from various spectroscopic measurements, e.g., n.m.r., electron spin resonance, dielectric relaxation, fluorescence, which allow the specification of rotational and translational diffusion processes in fair detail. For studies of transport properties at a lower level of resolution, the various hydrodynamic methods find extensive application. Indirectly these methods provide an indication of size and shape of diffusing species. The total charge is monitored by allowing the species to diffuse in the presence of an electric field, e.g., electrophoresis.

III. HYDRATION INTERACTIONS OF SMALL MOLECULE SPECIES

A. Ion Hydration

In any direct interaction (binding) involving a protein and an ion, or in a more general "electrolyte medium" effect on conformational stability, the ion must be treated as a solvated species which may undergo some modification during interaction with a macromolecule. Since the interaction therefore involves the *hydrated* ion and the *hydrated* macromolecule, some of the characteristic features of ion hydration are mentioned here. Another important aspect which involves ionic hydration is the stability of proteins towards changes in pH. The dissociation of acidic or basic residues is likely to be accompanied by considerable changes in their solvation states which in turn affect the conformational stability of the macromolecule. Ionic solvation states and energies are therefore likely to have a role in maintaining (or perturbing) biopolymer tertiary structures.

The Born model of ion hydration is based on the simple concept that the ion is a hard sphere of radius r_i^* and the solvent is a continuum of dielectric permittivity ϵ which is unaffected by the electrostatic field due to the ion. The standard free energy $\Delta G_{t_i}^{\ominus}$ associated with the transfer of an ion of species i from some reference state into a solvent is then given by Equation 1.

$$\Delta G_{t_i}^{\ominus} = (N z_i^2 e^2 / 2 r_i^*)(1 - \epsilon^{-1}), \qquad (1)$$

where z_i is the valence and e the electronic charge. Different methods have been proposed for evaluating r_i^*[7,8] and this is of course the crux of the problem, since ions are not hard spheres. However, self-consistent sets of r_i^* values for given series of ions, e.g., the alkali metal, or the halide ions, can be evaluated by means of which $\Delta G_{t_i}^{\ominus}$ (r_i^{*-1}) can be shown to be reasonably linear for z = 1,2, but noticeable deviations occur for z = 3. The shortcomings of the model become very apparent when we consider the thermodynamic functions derived from the temperature dependence of $\Delta G_{t_i}^{\ominus}$ e.g., the enthalpy of transfer $\Delta H_{t_i}^{\ominus}$. Krishnan and Friedman have shown quite conclusively that, even leaving aside water as a unique solvent, marked ion specific effects are observed in solvents such as dimethylformamide and methanol.[9] Various attempts to refine the Born model, e.g., by making allowance for the specific dielectric properties of an ionic solvation sphere,[10] have failed to provide credible models to account for the observed subtleties of ion–solvent interactions.

As soon as allowance is made for the *molecular* nature of the solvent in a general description of solvation, then the methods of classical electrochemistry become inadequate and statistical thermodynamic techniques must be employed to calculate molecule and ion distributions and interactions, but these too contain built-in assumptions and simplifications which limit their use. As a compromise, several composite or hybrid models have been proposed in which the properties of the primary ionic hydration sphere are rigorously calculated and the solvated ions are then treated by the Born model. Finally, some ingenious chemical approaches have been used to estimate the contribution of a solvation complex, e.g., $X^+.nH_2O$ to the observed thermodynamic properties of the solution. The difficulty here is that often the effects produced by ions, e.g., on the stability of proteins in solution, suggest that it is not the primary hydration sphere, but solvent perturbations further removed from the center of the ion which are responsible for the experimental observations.

In an important series of publications summarized in Reference 11, Friedman and his colleagues have developed a model for solvation which combines various aspects of the treatments outlined above but is not dependent on any given detailed molecular specification of the solvation sphere. It is based on a specification of the concen-

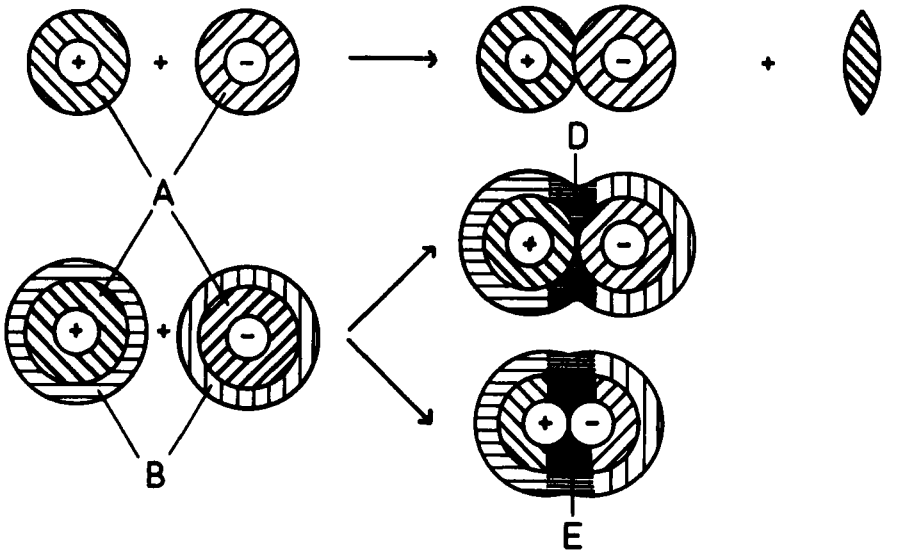


FIGURE 2. The interaction between solvated ions: (1) in terms of primary hydration spheres (A) only, showing destructive interaction with release of solvent which then relaxes to its usual unperturbed state, and (2) allowing for secondary hydration (region B): outer cosphere overlap (region D) and primary cosphere overlap (region E), giving rise, respectively, to solvent separated and contact ion pairs. (Adapted from Lilley, T. H., Raman spectroscopy of aqueous electrolyte solutions, in *Water – A Comprehensive Treatise*, Vol. 3, Franks, F., Ed., Plenum Press, New York, 1973, chap. 6).

tration dependence of the thermodynamic properties of an electrolyte solution in terms of ionic pair interactions. Figure 2 shows how two solvated ions may interact.* The process depicted corresponds to destructive interaction – some solvent is "squeezed" out and relaxes to its normal, unperturbed state; the corresponding free energy change A_{ij} can be calculated. (In the particular model under discussion it is assumed that the extent of the solvation sphere corresponds to one solvent molecular layer.) A_{ij} and its pressure and temperature derivatives can be related to the excess thermodynamic properties such as enthalpy, entropy, volume, compressibility, expansibility, and heat capacity. The interpretation of A_{ij} and its derivatives in terms of the molecular details of solvation relies to some extent on chemical intuition and evidence derived from nonthermodynamic measurements, particularly infrared, Raman, and n.m.r. spectroscopy. Thus Krishnan and Friedman differentiate between hydration shells in which water is oriented by ionic fields or other directional forces and water which is perturbed by the proximity of a solute (e.g., an alkyl group) where the effect cannot arise from directional solute-solvent forces.[11] The former group can again be subdivided into three types: (a) water held in the primary hydration sphere of a small cation, (b) water held in the primary hydration sphere of a small anion, and (c) water hydrogen bonded in the primary hydration sphere with ions containing -OH or R_3NH^+ groups.

The spectroscopic studies which must go hand in hand with the thermodynamic characterization of hydration processes fall into two categories: (1) investigations of changes in the ionic species, and (2) investigations of the effects induced by the solute on the spectrum of water. The application of infrared and Raman spectroscopy to the former is limited to the study of polyatomic ions, so that most available information on univalent ions is based on changes observed in the spectra of water. Although at the present time such information is still largely of a qualitative and comparative nature, nevertheless it does provide a fair degree of insight into the nature of the type of hydration sphere depicted in Figure 2.[13,14] For example, it is now possible to distinguish between states A and B in Figure 2, and recent nuclear magnetic

*The same argument can be applied to nonelectrolyte particles,[12] in which case the interaction potential would not contain an electrostatic term.

relaxation studies have characterized the orientation of water molecules in the fluoride ion hydration sphere.[15] Furthermore, the fundamental difference between "normal" ionic hydration and the interactions between water and alkylated ions is now well established,[16] and it is recognized that the relative abilities of different ions to perturb the orientations and the intra- and intermolecular vibrations of water provide the origin of the famous Hofmeister or lyotropic series[17] of ion-specific effects (see below). Finally, the probing of the diffusive (rotational and translational) motions of water under the influence of ions by nuclear magnetic and dielectric relaxation methods is providing information about the dynamic nature of isolated and overlapping hydration spheres. Just as the purely electrostatic Born model is inadequate to account for the thermodynamics of hydration, so the analogous Stokes-Einstein model relating ionic mobility and rotational diffusion to the bulk properties of the solvent medium has been found to be inadequate to account for the observed transport properties of ionic solutions in general and of aqueous solutions in particular.[18] Really startling deviations from the predicted ion transport behavior are observed in mixed aqueous solvents, e.g., H_2O - alcohol,[18] and the effects of such solvent systems on the properties of protein solutions are treated in Section VI. E.

B. Hydrophobic Effects

It is now generally realized that the hydrophobic interaction plays an important role in maintaining the conformational integrity of globular proteins and also appears to be implicated in the promotion of complex intermolecular structures, such as the double or triple helices encountered in proteins, nucleotides, and polysaccharides or in multi subunit protein structures.[19]

The origin of the hydrophobic effect derives from the interaction (or lack thereof) of water with apolar groups.[20] Thus aqueous solutions of apolar molecules are unique in that their thermodynamic properties are dominated by a *negative* excess entropy of solution. Experience has shown that the term "apolar molecule" can also be applied to monofunctional alkyl and aryl derivatives and even to ionic species containing substantial alkyl or aromatic residues, such as the long chain alkyl sulfates or quaternary alkylammonium cations.[21] All these molecular and ionic species have one feature in common: their thermodynamic solution properties are determined primarily by the number of CH_2 groups in the alkyl chains.

The diagnostic features of hydrophobic hydration (i.e., transfer of an alkyl group from an apolar solvent to water) are summarized in Table 1. Although the magnitudes of the various thermodynamic properties summarized depend primarily on the number of CH_2 groups, alkyl group conformation, i.e., normal, branched chain or cyclic, also contributes, but to a lesser degree; however, the nature of the polar functional group is unimportant.[22] Various spectroscopic examinations of aqueous solutions of hydrophobic molecules have shown that the effect of the solute molecule is largely to hinder the rotational motions of, and enhance the degree of hydrogen bonding between water molecules.[20] The solute molecule itself appears to be very little affected. It is also known that the alkyl derivatives under discussion promote the formation of ice clathrate structures. These are complex crystalline hydrates in which the water molecules form a hydrogen bonded lattice containing cavities of various sizes which accommodate the solute "guest" molecules.[23] The clathrate can be regarded as a first cousin of ice I, in that both structures are based on a hydrogen bonded 4-coordinated lattice with identical hydrogen bond lengths. The differences arise in the orientational details. Whereas the molecules in ice are truly tetrahedrally coordinated, in the clathrates slight deviations from the tetrahedral angle give rise to polyhedral cavities bounded by upwards of 12 faces. Figure 3 compares ice with a simple clathrate cavity and a complex 17-hedral cavity as found in the clathrate of tert-butylamine.

The thermodynamic and spectroscopic properties of dilute aqueous solutions of alkyl derivatives are consistent with the following picture:

1. The alkyl group is primarily responsible for hydrophobic hydration.

2. Hydrophobic hydration involves a rearrangement and/or reorientation of water molecules to create a suitable cavity capable of accommodating the apolar residue. Clearly such a process cannot be confined only to the primary hydration shell, but must involve water molecules further removed from the solute particle. Hydrophobic

TABLE 1

Characteristic Features of Hydrophobic Hydration and the Hydrophobic Pair Interaction. Standard Thermodynamic Properties $\Delta X^{\ominus}$ refer to the process: Alkyl-X (gas) → Alkyl-X (aq., infinitely dilute). X can be H, –OH, $-NH_2$, >NH, N,-O-, >CO. Thermodynamic properties $\Delta X_{H\phi}$ refer to the process 2-CH_2- (aq. infinitely dilute) → –CH_2 CH_2– (aq). According to the classical model of the hydrophobic interaction, $\Delta X^{\ominus}$ and $\Delta X_{H\phi}$ should have opposite signs.

Hydrophobic hydration expressed/mol CH_2		Hydrophobic intention expressed/mol pair CH_2	
$\Delta G^{\ominus} > 0$	800 J	$\Delta G_{H\phi} < 0$	~ –2.9 kJ[a]
$\Delta H^{\ominus} < 0$	4.2 kJ	$\Delta H_{H\phi} > 0$	105 J $molal^{-1}$ [b]
$T\Delta S^{\ominus} < 0$	5 kJ	$T\Delta S_{H\phi} > 0$	–
$\Delta Cp^{\ominus} > 0$	15 J deg^{-1}	$\Delta Cp_{H\phi} > 0$	–
$\Delta V^{\ominus} < 0$	~ –1.5 cm^3	$\Delta V_{H\phi} < 0$	≅ –0.4 cm^3 $molal^{-1}$ [c] +0.7 cm^3 [a]
$\partial\Delta V^{\ominus}/\partial T < 0$	~ –3 cm^3 deg^{-1}	$\partial\Delta V_{H\phi}/\partial T < 0$	
$\partial\Delta V^{\ominus}/\partial P < 0$	–	$\partial\Delta V_{H\phi}/\partial P < 0$	

[a]Calculated from the "classical" hydrophobic bond theory (Nemethy, G. and Scheraga, H. A., *J. Phys. Chem.*, 66, 1773, 1962.)
[b]From experimental enthalpy pair interaction coefficients (Franks, F., Pedley, M. D., and Reid, D. S., *J. Chem. Soc. Farad. Trans.*, in press, 1975.)
[c]From experimental volume pair interaction coefficients (unpublished data).

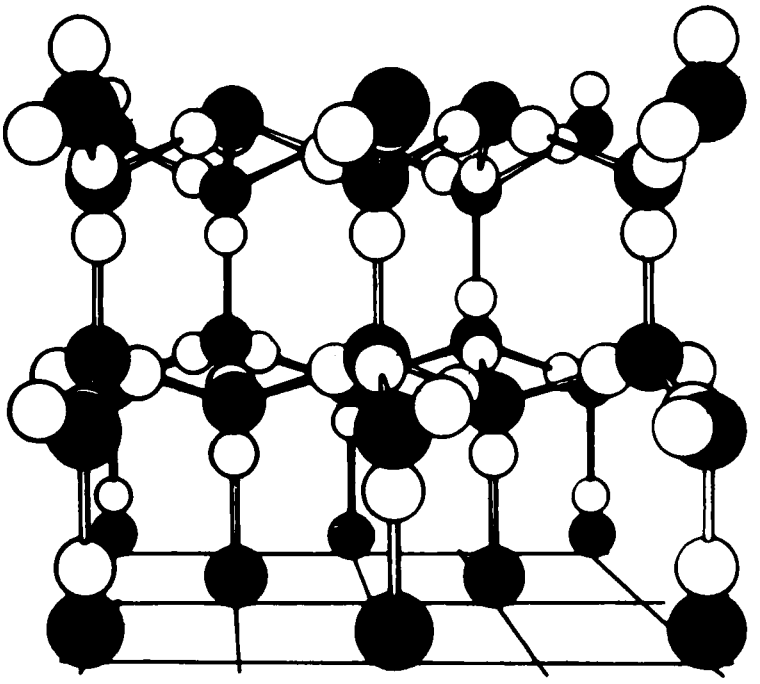
hydration can thus be regarded as a weak, long-range interaction. This point will be further developed in Section VI.E.

3. The formation of the hydrophobic hydration shell is an entropically unfavorable process; the water molecules can be regarded as "more structured" and slower moving than in bulk water. The large ΔC_p of solution indicates that such hydrophobic hydration structures are thermally very labile.

4. The observed P–V–T changes associated with hydrophobic hydration, e.g., negative excess volumes of solution, support the picture that the solute molecule partly occupies void volume which had already been available in the bulk water structure, and arises from the fact that water is not by any means a close-packed liquid.

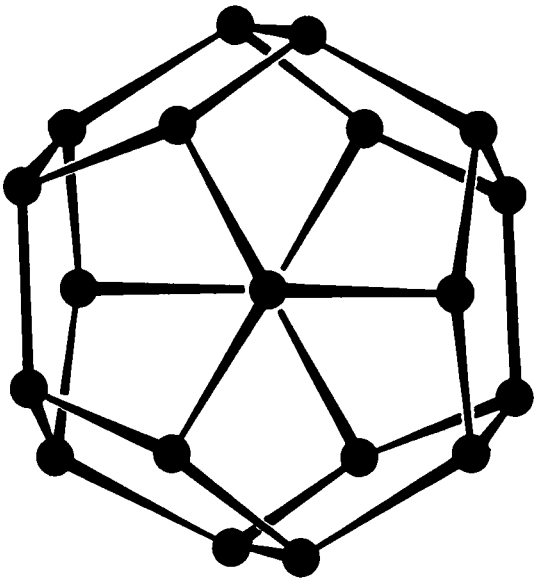
The above conclusions indicate that the hydrophobic hydration phenomenon must be intimately related to the energetics of the intermolecular hydrogen bonding in liquid water. One way of modifying this feature is through the substitution of D_2O for H_2O. Although such a substitution produces changes in all solvent interactions, these changes are particularly marked for hydrophobic hydration effects. As a consequence, wherever hydrophobic interactions provide a significant contribution to the conformational stability of a protein, then the substitution of H_2O by D_2O leads to dramatic changes in the observed conformational equilibria, in a similar direction as would be produced by a lowering of the temperature.

In recent years several attempts have been made to calculate the destabilizing contribution of hydrophobic hydration to the conformational free energy of proteins. The common basis for such calculations is the scaled-particle theory which divides the solution process into: (1) the formation of a cavity within the solvent, capable of accommodating a solute molecule, and (2) the introduction of the solute molecule into the cavity with an allowance for solute–solvent interactions. Although Pierotti[24] had some apparent success in applying the scaled-particle theory to aqueous solutions of alkanes (he was able to "predict" heats, entropies, and heat capacities of solution), his calculations were based on water as a system of



3A.

FIGURE 3. The geometrical arrangement of water molecules in (a) the ice lattice, (b) a pentagonal dodecahedron, characteristic of inert gas hydrates, and (c) a complex 17-hedral arrangement, as found for *tert.*-butylamine clathrate hydrate. Note that minor deviations from the true tetrahedral angle can give rise to large varieties of clathrate-like cavities, capable of accommodating solute molecules of different shapes and sizes.



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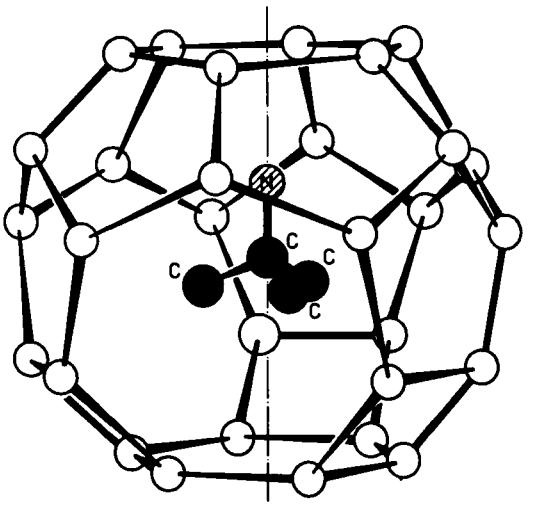


3C.

hard spheres. His work has since been elaborated on by several other workers.[25-27] Since the unique nature of water as a solvent depends largely on its interaction anisotropy, this type of approach, even if it provides some right numbers, must be suspect. A more recent and much more rigorous appraisal of the scaled-particle theory as applied to water has provided a much more detailed insight into the possible nature of hydrophobic hydration shells.[28] Figure 4 shows one such (idealized) arrangement of water molecules surrounding a spherical noninteracting particle. The water molecules fall into two categories – nearest neighbors (shown as dark circles) and more remote neighbors (shown as open circles). The former have a strong orientational bias, so that one of the four possible hydrogen bonding directions always points radially away from the solute particle; this is also a feature of the clathrate lattices. Stillinger suggests that on energetic grounds, the largest possible convex hydration cage might resemble a soccer ball pattern with 60 water molecules forming 12 pentagons and 20 hexagons.[28]

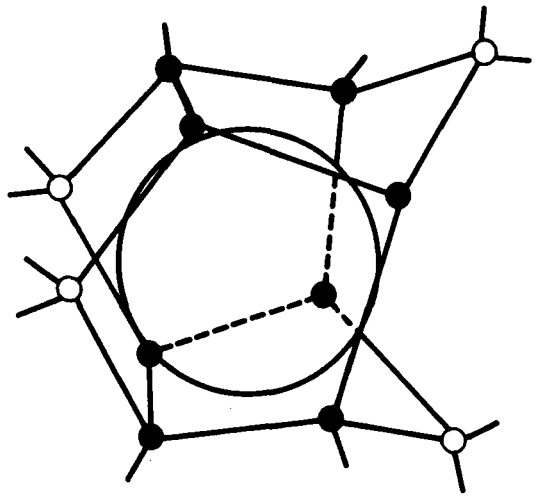


FIGURE 4. Typical convex water molecule cage enclosing a nonpolar molecule, consisting of 12 H_2O molecules arranged into four pentagons and two hexagons. The oxygen atoms can be classified into 8 nearest neighbors (•) and 4 next-nearest neighbors (o). Calculated by application of the scaled-particle theory to the formation of convex cavities in water. (See Reference 28.) (From Stillinger, F. H., Structure in aqueous solutions of nonpolar solvents from the standpoint of scaled-particle theory, *J. Solution Chem.*, 2, 141, 1973. With permission.)

Although the type of convex cage statistics used by Stillinger may eventually help to provide a better understanding of the hydrophobic element in protein conformational stability, the calculations so far performed are very much less sophisticated and are based on the concept of "accessible surface area" to describe the relationship between alkyl groups and solvent. In this section we are concerned with calculations performed on model hydrophobic compounds, but in Section IV the application of this approach to globular proteins is discussed. The "accessible surface area" can be regarded as the "area of the surface of a sphere of radius R on each point of which the center of a solvent molecule can be placed in contact with the atom (e.g., a hydrogen atom of a methylene group) without penetrating any other atoms of the molecule. R is the sum of the van der Waals radii of the atom and the solvent molecule".[29]

Reynolds et al. use the observation that $\Delta G_t^{\ominus}$ for the transfer of *n*-alkanes from their pure liquid state to an aqueous medium is a linear function of the hydrocarbon chain length[30] and combine this with published data for solvent cavity surface areas[31] calculated on the above basis.* The reader will note the similarity with the concept developed by Stillinger, but now the solvent molecule is once again explicitly treated as a hard sphere.

The alkane solubility s is expressed as:

$$RT \ln s = bA + C, \quad (2)$$

where A is the cavity surface area, b is the hydrophobic free energy per unit area, and C is a constant. A plot of Equation 2, using the most reliable alkane solubilities,[32] gives a straight line passing through the origin, with b = 88 J mol^{-1} $Å^{-2}$.

A similar approach has been used to estimate the hydrophobic free energies for a series of amino acid residues.[33] The "side chain hydrophobicity" is obtained from $\Delta G_t^{\ominus}$ measurements by subtracting the glycine value from that of the amino acid under study.[34] The "hydrophobicity" is a linear function of the size of the alkyl groups with a slope b = 92 J mol^{-1} A^{-2}. Residues with similar accessible surface areas, but also possessing a polar group, have hydrophobicity values which are lower by 4.2 kJ mol^{-1}, but it is not clear how the concept of accessible surface area may need to be adjusted to allow for hydrogen bonding between solute and water.

*A word of warning is required here. If the disposition of water molecules surrounding an alkyl chain resembles the clathrate geometry, then the calculated cavity surface areas arrived at on the basis of close packing must be in error.

Since, from the above model calculations, the exposure of alkyl groups to water requires a free energy expenditure of ~3.6 kJ (mol CH_2)$^{-1}$,[30] the partial reversal of this process, i.e., the substitution of solute-water by solute–solute contacts, should be thermodynamically favorable and take place spontaneously. This process, involving the aggregation of alkyl residues in aqueous media, is termed the hydrophobic interaction and is believed to be one of the main contributing factors in promoting the conformational stability of proteins.[35] It also provides the driving force for the micellization of amphiphiles,[36] the aggregation of dyestuffs,[37] the stability of aqueous phospholipid bilayer structures,[38] and for many binding processes involving biopolymers.

If the simple picture of a solute–solute contact with concomitant "squeezing-out" and relaxation of some of the hydration shell water correctly represents the hydrophobic interaction, then the associated thermodynamic functions $\Delta X_{H\phi}$ would have the opposite sign from those describing the hydration process. Table 1 includes a summary of the presently available experimental information and, while it is clear the $\Delta G_{H\phi}$ is indeed negative, volumes and heat capacities in particular suggest that the currently accepted model for the hydrophobic interaction which is based almost completely on free energy of transfer data cannot correctly account for the limiting concentration dependence (i.e., the second virial coefficient) of some of the derived thermodynamic properties of aqueous solutions of model hydrophobic solutes. This discrepancy is further emphasized by the vibrational[39] and diffusional[40] properties of water molecules under the influence of increasing concentrations of alkyl residues. All available spectroscopic evidence suggests that with increasing concentration of alkyl groups the rotational motions of water molecules become restricted and the degree of hydrogen bonding further enhanced over and above the values characteristic of the infinitely dilute solutions. At any given temperature there is a critical concentration c* of CH_2 groups beyond which the observed effects become reversed and then the further addition of hydrophobic residues causes water to lose those properties which make it unique as a solvent. The significance of c* of hydrophobic additives which are said to promote protein unfolding is discussed in some detail in Section VI.E.

C. Specific Hydration Effects

Water is a highly polar molecule (for many purposes it can be represented quite adequately as a regular tetrahedron with two positive and two negative charges at the vertices) which can participate in the formation of up to four hydrogen bonds, and it is to be expected that direct hydrogen bonding interactions with polar solutes contribute significantly to hydrated states of polar molecules. Since the sp^3 hybridized orbitals of an oxygen center are localized, hydrogen bonding interactions with the solvent are likely to be highly orientation specific. This has been confirmed by comparative studies on a series of sugars. It was found that the hydration interactions of axially and equatorially placed OH groups exhibit significant differences, especially at low temperatures.[41,42] This directional specificity is in contrast to ionic and hydrophobic hydration both of which are orientationally nonspecific.

In proteins the functional groups which can participate in specific hydrogen bonding with the solvent include the backbone peptide repeat unit shown in Figure 5, and the various hydrogen bonding centers on polar amino acid side chains, e.g., OH in serine, threonine, hydroxyproline and tyrosine, and *undissociated* acidic or basic groups, as in glutamic acid, aspartic acid, arginine, lysine, histidine, and tryptophan. The detailed hydration structures of ionized groups such as $-COO^-$, $-NH_3^+$, $-NH_2^+$ are likely to be quite complex,[43] combining the requirements of the radial Coulombic field with the orientational specificity of the solute-water hydrogen bonds.

Model compounds for studies of the hydration characteristics of polar sites in proteins have included amides, oligopeptides, and homopolypeptides. Since infrared and Raman active vibrational

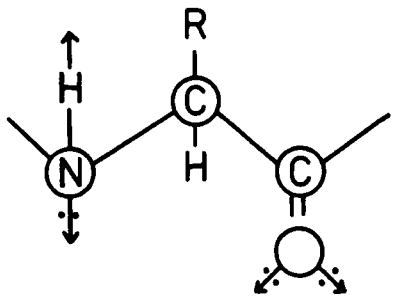
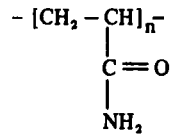


FIGURE 5. Possibilities of specific hydration interactions by peptides, showing the three proton accepting, and one proton donating sites.

modes of the H_2O molecule are very sensitive to local perturbations, these spectroscopic techniques have been employed to study hydrogen bonding between water and polar sites on peptides.[44,45] However, just because liquid water is so extensively hydrogen bonded, its infrared spectrum is very complex and there is as yet no general agreement about the correct assignment of all the observed bands. One useful method of simplifying the spectra is based on the addition of low concentrations (~10%) of D_2O so that the observed species is HDO in which the frequencies corresponding to the OD and OH vibrations are far enough removed for them not to exhibit coupling.[47] However, even this device does not always yield very informative solution spectra and, therefore, most of the available data refer to solid peptides with low water content, so that the interaction of peptide groups with isolated vicinal water molecules is in fact studied.[44] The experimental problems associated with spectroscopic studies of protein and peptide hydration *in solution* are discussed in some detail in Reference 6.

The available evidence strongly suggests that in aqueous media, peptide–solvent hydrogen bonding is not preferred to solvent–solvent hydrogen bonding, or indeed to peptide–peptide bonding. The spectroscopic studies on model amides in various solvents have also demonstrated that the strength of the intermolecular -C=O...H-N- bond varies inversely with the hydrogen bonding potential of the solvent and that in water the enthalpy of formation of such a bond is essentially zero.[48-50] Also the transfer of a peptide bond from an apolar to an aqueous environment is an exergonic process. The above facts must therefore raise the question of the role of peptide hydrogen bonding as a contributing factor to the conformational stabilities of proteins in solution.

Several ingenious ways of probing the nature of the solvated amide group have recently been described by von Hippel and his colleagues.[51-53] A summary of the results and their relevance to biopolymer conformational stability is given in Reference 54. The interaction of neutral salts with a series of polyacrylamide (I) gels was determined by recycling chromatography.



(I)

By comparative measurements on polystyrene columns it could be shown that the interaction must occur with the amide group and that ions are neither attracted to nor repelled from the hydrophobic hydration shell surrounding the nonpolar groups. It is also interesting to note that no ion binding could be observed on cross-linked dextran columns, i.e., binding is specific to the $-CONH_2$ residue. This may seem to be a profound statement, but reference to Figure 5 shows that the amide group possesses several distinguishable binding sites; nothing is yet known about the structural or stereochemical aspects of the different amide-water-ion complexes. Since the polar–nonpolar group ratio of I resembles that of an average exposed residue in a globular protein, $\Delta(\Delta G_t^{\ominus})$, corresponding to the transfer of a typical residue from water to solutions of denaturing salts can be evaluated; single ion binding constants and derived thermodynamic properties have been calculated, and Table 2 summarizes some of the results. The order in which the ions facilitate the destabilization of proteins, as measured by the model binding experiments, closely corresponds to the well-known lyotropic series. This was first discussed by Hofmeister[17]* as a result of measurements of the concentrations of electrolytes required for the precipitation of serum globulins. He showed that the order of increasing effectiveness as precipitants is as follows:

$$\text{Cations: } Li^+ > Na^+ > K^+ > NH_4^+ > Mg^{++}$$
$$\text{Anions: } SO_4'' > HPO_4'' > OAc' > \text{citrate} > \text{tartrate} > Cl' > NO_3' > ClO_3'.$$

Over the years this series has been supplemented, e.g., I', ClO_4', and CNS' can be added at the end of the anion series, and the alkylammonium ions form another group such that

$$Me_4N^+ > Et_4N^+ > Pr_4N^+ >> Bu_4N^+$$

*As is so frequently the case, the lyotropic series which bears Hofmeister's name was really discovered by someone else, and Hofmeister in his publications acknowledges this fact.

TABLE 2

$\Delta(\Delta G_t^{\ominus})$ Measures the Ionic Contributions Towards the Destabilization of a Folded Protein Compared to the Thermally Unfolded State. Results Refer to 1 *M* Concentration of Ions and Have Been Arrived at by Equating the Effects of Na^+ and Cl^-

Ion	$\Delta(\Delta G_t^{\ominus})$ J (mol residue)$^{-1}$
K^+	+ 5.1
Na^+	- 7.7
Rb^+	- 12.6
Cs^+	- 24.9
Li^+	- 24.9
Mg^{++}	-105
Ca^{++}	-117
Ba^{++}	-121
F^-	+ 77
SO_4^{--}	+ 34.8
Cl^-	- 7.7
Br^-	- 73
NO_3^-	- 92
SCN^-	-130
ClO_4^-	-168

with the first member corresponding to Na^+ in its effectiveness.

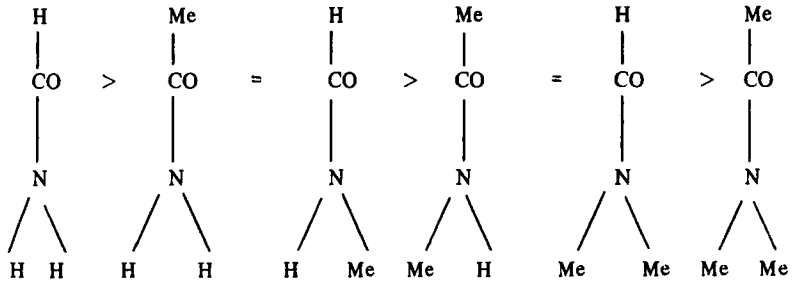
After speculating on possible connections between the protein precipitating potential of ions and their diuretic action, Hofmeister advanced the remarkable suggestion that all the observed ion specific effects were probably manifestations of differences in the same fundamental property, namely the power of ions to bind water.

It is a chastening thought that almost 90 years later there has been very little advance in our understanding of the phenomena which form the basis of the lyotropic series. It has, however, been established that the two ionic series are operative in promoting salting-out of nonelectrolytes, regulating order-disorder transitions of biopolymers, stabilizing (or destabilizing) lyophobic sols, etc. The original approaches adopted by von Hippel may turn out to be a significant advance towards the solution of the intractable problems posed by the Hofmeister series.

The differential ionic effects referred to above and shown in Table 2 indicate that ions might be excluded from the polyacrylamide primarily by their relative inability to compete for water molecules directly bound to the amide dipole. NaF is almost completely ineffective in competing for amide bound water and therefore a comparison of the column volume available to $^1H^3HO$ and NaF serves as an index of hydration of the amide group. This turns out to be $\sim 2H_2O$ per amide group, in good agreement with the estimates arrived at on the measurement of "unfreezable" water.[55] For uni-univalent salts binding is energetically favorable ($\Delta H_t^{\ominus} < 0$), but this is more than offset by an unfavorable entropy, indicating a net ordering.

The introduction of methyl groups near the amide residue leads to interesting changes in the affinities of the dipoles for salts. Thus the affinity decreases in the following order:

formamide > acetamide > N-methylacetamide > N,N-dimethylacetamide
= N-methylformamide = N,N-dimethylformamide



However, methyl substituents at different sites affect ion binding to different extents. Once again the order in which ions are affected corresponds to the Hofmeister, or lyotropic series, and seems to originate in the modulation of binding by the vicinal apolar groups. Binding to the "ideal" amide dipole (formamide nearly fulfils this criterion) is nonspecific. The methyl groups do not participate

directly in the binding process but seem to exert their influence through their hydrophobic hydration spheres which interact with the "solvent structure" associated with the hydrated amide-ion complex. It seems to us a reasonable hypothesis that the behavior here discussed, i.e., in alkyl substituted amide-water-electrolyte systems, should depend to a large extent on the compatibility or incompatibility of the different hydration states of the various species, i.e., ionic, hydrophobic, and specific hydrogen bonding, as described in this and the preceding sections.

Various "explanations" of the Hofmeister effects have been proposed. For instance, it has been suggested that the basis for the anionic series lies in the varying abilities of ions to act as proton acceptors in hydrogen bonding with the solvent.[56] However, von Hippel makes the telling point that whatever interpretation is favored by individual investigators, it always reduces to competitive water reorganization by ions, dipoles, and hydrophobic groups, and an understanding of the ubiquitous Hofmeister effects awaits "an unravelling of the equilibrium hydration structures of various ions, dipoles and nonpolar groups and an appreciation of the thermodynamic consequences of these structures."[54]

It is in this context that we must examine the solvent involvement in the maintenance or disruption of the conformational integrity of proteins.

IV. FACTORS WHICH CONTRIBUTE TO THE CONFORMATIONAL INTEGRITY OF PROTEINS

The interrelationships between protein and solvent may be examined at different degrees of molecular resolution, and it must immediately be pointed out that high resolution is not always synonymous with superior information. On a low level of resolution we may use Lumry's representation[57] (Figure 6) which views the protein as a giant homogeneous sphere surrounded by a number of distinguishable solvation shells which are characterized by the degree of dynamic and structural perturbation suffered by the water molecules included in these shells. Any such perturbations will become attenuated as the distance from the perturbing group increases, but

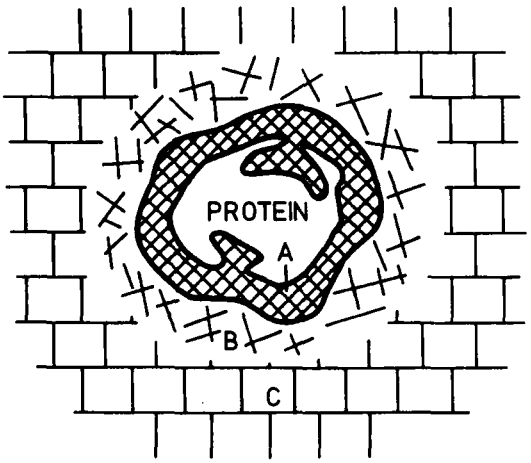


FIGURE 6. Low resolution representation of protein hydration. The protein, although of irregular shape, is conceived as a hydrodynamic sphere with A – a primary hydration shell in which the molecular orientations are dominated by the polar (and apolar ?) protein sites, and B – a secondary hydration shell where these orientational effects are attenuated and which eventually merges into region C – unperturbed water. (Adapted from Lumry, R., *Participation of water in protein reactions*, in The Mechanism of Energy Transduction in Biological Systems, *Ann. N.Y. Acad. Sci.*, 227, 471, 1974. With permission.)

since we are dealing with a liquid system, there must be some coupling even between water molecules adjacent to the protein and those many molecular diameters removed.

Lumry points out that most of the experimental techniques normally employed in studies of protein hydration (for a review see Reference 6) are able to monitor hydration effects only in the A shell which is probably responsible for solubility properties but unlikely to change dramatically on unfolding of the protein. Circumstantial evidence mainly of a thermodynamic nature suggests that it is the second B hydration shell which plays an important role in determining the conformational stability.

On a more detailed basis the conformational free energy G_N of a folded native protein can be considered as the sum of various contributions, some of them arising from intrinsic, intramolecular sources, and others from solvation effects. At the present time too little is known about the details of most of these interactions for native conformations of proteins, and hence G_N to be calculated with any degree of confidence.* Much of the

*This review deals primarily with the role of the solvent in the N → D process, and we do not therefore discuss the details of such calculation procedures or appraise the more indirect methods of evaluating ΔG. We would, however, question the value of "vacuum" potential energy calculations as a means of arriving at G_N.

empirical information about G_N has therefore been obtained indirectly from studies of reversible denaturation or unfolding of proteins by various means, and it is usually assumed that the denatured or unfolded state can be considered as a random coil with a characteristic free energy, G_D, and that the reversible D state is therefore independent of the method used for perturbing the folded state, i.e., $\Delta G_{N\rightarrow D}$ is a true equilibrium property for a given protein and is independent of the path N→D. This approach is particularly favored for unfolding experiments with urea or guanidinium hydrochloride (GuHCl).[58] Moreover, unfolding is usually regarded as a two-state, "all-or-none" process although careful experimental studies have, from time to time, indicated complex kinetic processes and transient intermediates.[59]* The evidence supporting the view of the D state as a random coil is also not quite as clear cut as some workers tend to suggest.[60] If the term, ("random coil") is meant to signify that all residues are exposed to the solvent, then the careful experiments of Tanford and his associates indicate that in concentrated urea or GuHCl, globular proteins behave in such a manner.[58] If, on the other hand, the term "random coil" is meant to imply, as it does in the case of synthetic polymers, an absence of even short-range order with complete rotational freedom about the backbone bonds, then there is mounting evidence against such a model, even in solutions of urea or GuHCl.[61,62] As to the postulate that all reversible unfolding processes, e.g., induced by temperature, urea, alcohol, lead to the identical "random coil" D state, we believe that the available evidence is overwhelmingly against such a possibility (see Section VI.E).

It seems to us that the term "random coil", as applied to proteins may need redefining, certainly a "denatured" protein containing regions of α-helix joined by flexible linking sequences would behave in solution as a "coil", when examined by hydrodynamic techniques; it would not, however, behave as a "random coil" by the criteria of complete rotational freedom, as monitored, for instance by n.m.r. or chiroptical methods.

In the following paragraphs the various contributing factors to $\Delta G_{N\rightarrow D}$ are reviewed, with particular emphasis on the solvent contribution. Considering first of all the intrinsic properties of the polypeptide chain, it is reasonable to postulate that although an unfolded protein may retain some internal structure, it must possess a greater degree of flexibility in the peptide backbone than the native state. Estimates of the conformational entropies associated with such increased rotational freedom range from 8 to 24 J per degree per unfolded residue,[35,48,49,63,64] and since the protein contains many hundreds of residues, this conformational entropy must provide a large contribution to the free energy of destabilization of the N state.

Intrapeptide hydrogen bonds have also been implicated[35,48] as a factor contributing to the stability of the N conformation, and estimates of the enthalpy of rupture of a peptide–peptide hydrogen bond have been given, ranging from 0 to 8 kJ mol^{-1}.[49,63] As pointed out in Section III.C, however, comparative studies on model amides in aqueous and nonaqueous solvents have suggested that the peptide–peptide hydrogen bond probably provides very little in the way of a stabilizing contribution to the native conformation.[64] Bello et al.[65] has, however, drawn attention to the cooperative element in peptide secondary structure, so that the small molecule analogue may well not be a valid model for intrapeptide hydrogen bonding. The fact is of course that internally hydrogen bonded structures do exist in proteins and model peptides. However, the sensitivity of such structures to pH, temperature, neutral salts, or organic additives suggests that amino acid side chain, rather than backbone interactions, may well be primarily responsible for the promotion of such ordered structures.[66]

The importance of surface energy contributions to the stability of a given protein was first discussed by Kauzmann,[35] and subsequent developments of the concept of hydrophobic interactions and hydrophobic hydration[67,68] have stimulated theoretical studies into its importance. Thus Bigelow in a classic publication attempted to define the concept of hydrophobicity in terms of total exposed hydrophobic surface.[69] His definition of the calculated average hydrophobicity $H\phi_{av}$ was based on Tanford's $\Delta G_t^{\ominus}$ data for amino

*There exists a large body of information about the conformational properties of homopolypeptides; these synthetic polymers have often been invoked as models for globular proteins. Although the conformational behavior of polypeptides is a fascinating field of study *per se*, we feel that it lies beyond the terms of reference of this review and the properties of homopeptides are referred to only where they appear of direct relevance to the discussion of proteins.

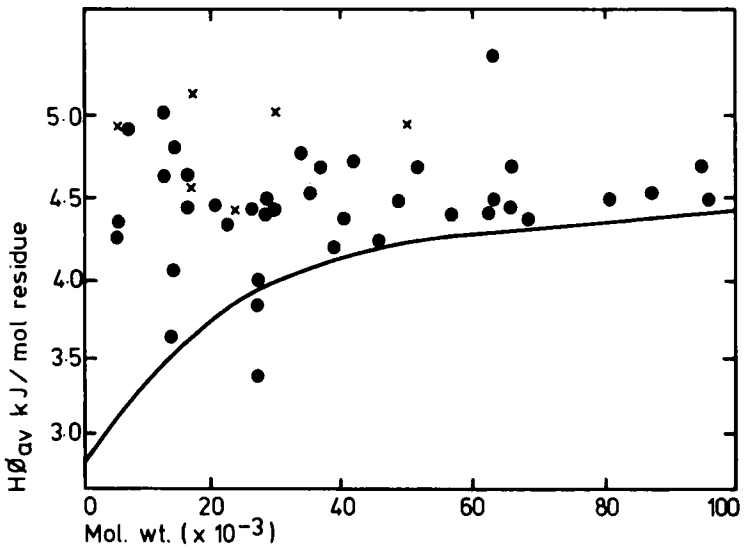


FIGURE 7. Average hydrophobicities, $H\phi_{av}$, of globular proteins as a function of their molecular weights; crosses refer to associating proteins (i.e., high $H\phi_{av}$). The curve shows the lower limiting $H\phi_{av}$ which is compatible with a globular conformation. Approximately 50% of globular proteins have $H\phi_{av}$ = 4.6 kJ (mol residue)$^{-1}$. (Adapted from Bigelow, C. C., On the average hydrophobicity of proteins and the relation between it and protein structure, *J. Theor. Biol.*, 16, 187, 1967.)

acids,[70]* and he was able to show that for over 150 proteins studied, $H\phi_{av}$ lay between 4.2 and 5 kJ (mol residue)$^{-1}$. Figure 7 shows the relation between $H\phi_{av}$ and the molecular weight. The curved lower limit shows that a protein must have a certain minimum $H\phi_{av}$ to be stable in a globular configuration. The application of the above concept, to fibrous proteins, where all side chains are exposed to the solvent, is not immediately clear; however, $H\phi_{av}$ values fall below 4.6 kJ, except for the elastins.

An interesting extension of the $H\phi_{av}$ concept deals with the comparison of thermal stabilities of homologous proteins derived from different species,[71] e.g., fish, mammalian and avian myosin. It appears that for very similar $H\phi_{av}$ values, considerable ranges of thermal stability are observed, so that the constancy of the hydrophobic portion of conservative proteins may be required for the stabilization of the tertiary structure and hence the function of the protein, but that the thermal stability cannot be attributed simply to the polar/nonpolar group ratio.

The concept of "accessible surface area" in calculations of hydrophobicity has been defined in Section III.B. The principle was applied quantitatively by Lee and Richards[29] to describe the relationship between protein and solvent. For the purposes of calculation the positions and orientations of the atomic groups of the protein molecule in solution were equated to those in the crystalline protein; the total accessible surface area exposed to the solvent was then computed. Shrake and Rupley[72] further refined the technique and calculated the degree of exposure of the protein to solvent. Figure 8 illustrates the degree of exposure of backbone and side chain atoms for lysozyme and Table 3 lists the total areas exposed in the native and unfolded states of the enzyme and of its complex with the hexasaccharide substrate. The data show that a large fraction of the total surface of native, as well as denatured globular proteins, consists of nonpolar atoms; these constitute 53 and 60% of the lysozyme surface in the native and unfolded states, respectively. A relatively high proportion (65%) of the nonpolar surface of the

*These data can only be accepted with the following reservations: (1) they refer to the transfer from ethanol to water, i.e., ethanol is equated with the interior environment of a protein, and (2) they refer to infinitely dilute solutions of amino acids, whereas during the unfolding of a protein interactions between neighboring side chains and between side chains and vicinal polar peptide groups are likely to modify the solvation effects.[52]

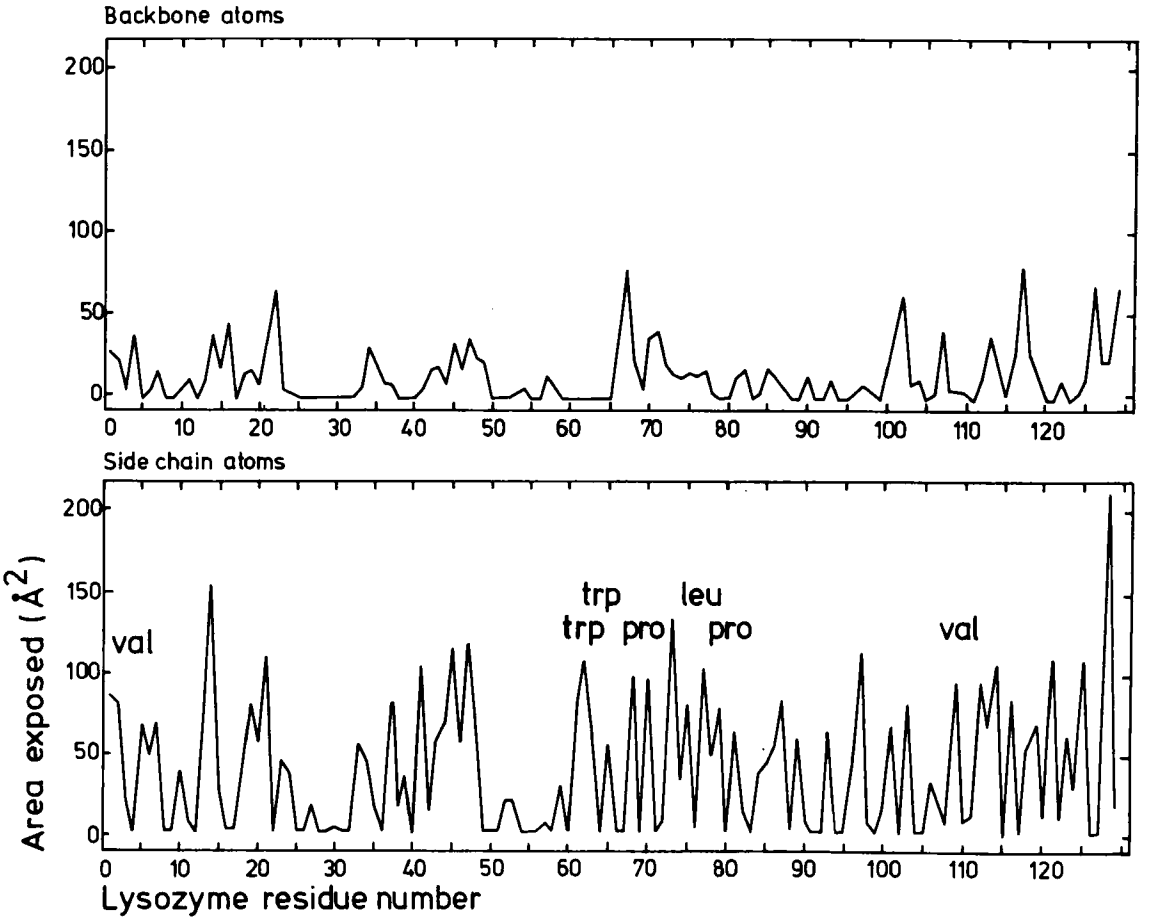


FIGURE 8. Area exposed to solvent by backbone (top) and side chain (bottom) atoms of residues in native lysozyme; some exposed apolar residues are indicated. (Adapted from Shrake, A. and Rupley, J. A., Environment and exposure to solvent of protein atoms in lysosome and insulin, *J. Mol. Biol.*, 79, 351, 1973.)

TABLE 3

Exposed Surface (Å²) of Lysozyme in the N and D States and its Hexasaccharide Complex

	Total	Polar	Charged*	Apolar†	Backbone	Side chain
A. Total						
D	21,723	6,175	2,466	13,082	5,840	15,884
N	6,583	1,811	1,261	3,511	1,599	4,984
Complex	5,919	1,586	1,128	3,205	1,462	4,457
B. Differences						
D – N	15,140	4,364	1,205	9,571	4,241	10,900
N – complex	664	225	133	306	137	527

*Includes groups that bear a charge at $0 < pH < 12$.
†Includes groups that contain only apolar atoms, and trp, met, and cys.

Adapted from Shrake, A. and Rupley, J. A., Environment and exposure to solvent of protein atoms in lysozyme and insulin, *J. Mol. Biol.*, 79, 351, 1973.

native protein derives from nonpolar atoms that are part of a polar or charged side chain, e.g., glu or lys, and it is implicitly assumed that the hydrophobicity of such methylene groups is not in any way affected by an adjacent polar or ionizable group. The extent to which nonpolar surface accessible in the denatured state is buried upon folding is two or three times greater for nonpolar residues than for polar residues. It is interesting to note that the net change in exposed nonpolar surface upon unfolding only constitutes 7% of the total protein surface; this implies that only relatively few peptide residues need to be exposed to the solvent in order to render the native conformation of the protein unstable.

The computations of Sharke and Rupley[72] are based on a solvent van der Waals radius of 1.4 Å, compared to 1.8 Å used by Lee and Richards, and the cavities within the lysozyme structure claimed by the latter authors could not be substantiated. However, the data do confirm the proposal of Lee and Richards that the density of side chain packing with the native protein is by no means uniform.

The calculations indicate that the carbonyl oxygen atoms are major contributors to the exposed surface of the polypeptide backbone; about half of the carbonyl oxygen atoms are exposed to the solvent in both helical and nonhelical regions. Glycine is one of the residues whose exposure is least affected by the folding process which accords with the proposal that gly lies on bends in the chain and on the molecular surface. Proline also is more exposed than might be expected from its nonpolar character, and this again may explain its participation in the bends of the peptide chain. Although pro is often classified with the nonpolar amino acids, it is in fact extensively hydrated.[73]

The hydrophobicity of a protein residue is now clearly seen to be related to the surface area of the residue but the nature of the relationship between $H\phi_{av}$ and surface energy is not apparent. The free energy change arising from structural changes in water produced by the removal of a nonpolar residue has been quoted as 3.6 kJ (mol CH_2)$^{-1}$ at 25°C,[30] with a marked temperature dependence. Attempts have also been made to quantify the water contribution to the hydrophic interaction by consideration of the loss of water surface free free energy, $-\gamma dA$ where dA is the change in surface area and γ the interfacial energy.[74] With γ = 50 erg cm^{-2} corresponding to a water/hexane interface, γdA is calculated as -4.6 and -11.8 kJ (mol pair)$^{-1}$ for gly-gly and diala-diala contacts, respectively. On a similar basis, the interfacial free energy of ribonuclease is estimated to be ~840 kJ mol^{-1}.[75] It seems quite evident that a reduction in the total protein/water interfacial area will be thermodynamically favored; this is also true for other macromolecules, e.g., polynucleotides.[76]

The above evidence does not of course explicitly account for the nature of the structuring effect (hydrophobic hydration) exerted by a nonpolar group on the neighboring water molecules. Once again using surface chemical considerations, Girifalco and Good[77] have examined the interfacial energies γ_{12} of a variety of interfaces:

$$\gamma_{12} = \gamma_1 + \gamma_2 - 2\sqrt{\gamma_1^d \gamma_2^d},$$

where γ_1 and γ_2 are the interfacial energies of the pure phases, and γ_1^d and γ_2^d are the dispersion force contributions to those energies. Significantly, only the long-range dispersion forces are capable of crossing an interface and exerting an attractive effect upon the components of the adjoining phase. Since the surface energy of a nonpolar material is almost totally due to dispersion forces, it becomes possible to determine the force exerted by a hydrocarbon on an adjacent water surface, namely $\gamma_1\gamma_2^d$, where γ_1 is the surface energy of the hydrocarbon and γ_2^d is the dispersion component in the surface energy of water, ~23 erg cm^{-2}.[78] Taking γ_1 as 30 erg cm^{-2}, and the difference in available surface area between val and leu as corresponding to dA for a methylene group, then the attractive energy exerted across the interface by the apolar group on the water is of the order of 2.3 kJ (mol CH_2)$^{-1}$; if each exposed CH_2 group is adjacent to perhaps 2 water molecules, then this implies a very weak orienting force, of the order of 1 kJ mol^{-1}, consistent with the properties of hydrophobic hydration spheres.

It is evident, however, that for the N→D transition of a globular protein the free energy change due to the hydrophobic interaction alone is insufficient to account for the stability of the N conformation.[60,79] Goldsack and Chalifoux, by considering the interior of the protein to approximate to an oil drop,[35] have pointed out that a contribution would be expected from a free energy of mixing term, if the hydrophobic residues

are assumed to be randomly mixed in the protein interior.[80] Table 4 lists the free energies of transfer and mixing for a number of proteins and it appears that the free energy of mixing term is of comparable magnitude to that arising from hydrophobic effects. While a free energy of mixing contribution to $\Delta G_{N\rightarrow D}$ may be justifiable, this can hardly assume the ideal, statistical value, since the apolar residues in the interior of the protein can hardly mix ideally, being individually attached to the rather rigid peptide chain. The estimates for $-\Delta G^M$ in Table 4 therefore constitute maximum values and are based on a model which can bear but little relation to the actual physical nature of a protein.

The above discussion leads to the expectation that the reversible N→D transition of a protein, particularly a globular protein, should be accompanied by considerable changes in the total exposed surface area of the macromolecule, and therefore also by appreciable changes in the volume, ΔV, of the biopolymer. This does not appear to be the case: for the reversible unfolding of ribonuclease at pH 4, ΔV ranges from -45 to -5 $cm^3\ mol^{-1}$, corresponding to a temperature range of 5 to 50°C.[81] It further appears that ΔV can be positive or negative,[82,83] implying that the observed ΔV arises from a difference between two or more volume changes of different sign. Model compound studies suggest that the exposure to water of a hydrophobic bond should result in ΔV = -20 $cm^3\ mol^{-1}$ of aliphatic side chains, with smaller negative values for aromatic side chains; thus, it has been suggested that protein unfolding should be accompanied by a massive negative contribution to ΔV,[75] due to exposure of hydrophobic side chains to the aqueous environment. This has been estimated as $> 1\ l\ mol^{-1}$. Since other contributions to ΔV are also negative, e.g., the interchange of peptide–peptide for peptide–water hydrogen bonds[83] (~-2 $cm^3\ mol^{-1}$ peptide), electrostrictive effects (up to ~-150 $cm^3\ mol^{-1}$ for ribonuclease),[84] this suggests that either some unknown process is involved which contributes in a positive manner to ΔV, or the basis on which model calculations are performed requires reexamination.

TABLE 4

Calculated Free Energy Changes of Mixing and Transfer of Apolar Groups for Proteins

Protein	Free energy of transfer $-\Delta G_t^{\ominus}$ (kJ)	Free energy of mixing $-\Delta G^M$ (kJ)
Amylase	4.63	4.97
Cytochrome C	4.65	4.54
Hemoglobin	4.72	4.60
Insulin	4.75	4.72
Lysozyme	4.40	4.93
Myoglobin	4.60	4.31
Myosin	4.14	4.13
Tropomyosin A	3.56	3.41
Tropomyosin B	3.36	3.25

From Goldsack, D. E. and Chalifoux, R. C., Contribution of the free energy of mixing of hydrophobic side chains to the stability of the tertiary structure of proteins, *J. Theor. Biol.*, 39, 645, 1973. With permission.

A possible basis for such a reappraisal is the computation of Shrake and Rupley[72] that, in the case of lysozyme, the proportion of apolar group/water interface changes from 53 to 60% of the total exposed surface during the N→D transition. This relatively small change in the extent of nonpolar surface area suggests that only small changes in volume due to hydration of hydrophobic groups occur during denaturation. Since the contribution to ΔV from aromatic side chain residues is $< -20 cm^3\ mol^{-1}$, it appears that the N→D transition occurs with the exposure of relatively few nonpolar residues. The data of Aune and Tanford[85] suggest that the exposure of a single buried aromatic side chain contributes about 12.5 kJ mol^{-1}, with the total $\Delta G_{N\rightarrow D}$ being of the order of 40 – 80 kJ mol^{-1} for lysozyme; this implies that denaturation involves the exposure of only 4 or 5 such residues. This would only contribute a volume change of ~ -50 to -100 $cm^3\ mol^{-1}$ to $\Delta V_{N\rightarrow D}$ and not values of the order of liters mol^{-1}, as has been suggested.

The complex behavior of ΔV and the experimental problems (e.g., time-dependent effects,) inherent in its determination have been well illustrated by Katz and his colleagues.[86-88] One very important consideration is the manner by which the N→D process is achieved, i.e., by temperature, pressure, pH, salt, organic denaturant, etc. Thus in the case of the pH induced unfolding of serum albumin, the dominant process contributing to ΔV is the normalization of 60 COO^- groups/10^5 g protein.[88]

One of the most incisive analyses of volumetric data, in this case ΔV accompanying the unfolding by GuHCl, has been performed by Lee and

Timasheff.[89] The results are based on the hypothesis that unfolding is due to binding of the third component (GuHCl), while its displacement of water presumably results from the A hydration shell (see Figure 6). A preferential interaction parameter is used to describe this process. (We shall return to the validity of this treatment in Section VI.A.) ΔV is calculated for the transfer of proteins from the aqueous native state to the hypothetically completely unfolded state in 6*M* GuHCl. No hydrophobic contribution needs to be invoked, and the calculated ΔV_t values are all found to be fairly small (see Table 5). By averaging ΔV_t over the total number of residues, admittedly a rough and ready procedure, ΔV_t per residue varies from zero to values rather larger than can be accounted for in terms of α-helix→coil and β-pleated sheet→coil transitions only.

Most of the experimental information about ΔV derives from densimetric or dilatometric measurements and is therefore confined to low pressures (usually atmospheric pressure). An alternative method of determining ΔV relies on the relationship:

$$(\partial \Delta G/\partial P)_T = \Delta V \qquad (3)$$

analogous to

$$(\partial \Delta G/\partial T)_P = -\Delta S$$

TABLE 5

ΔV_t for the Transfer of Proteins from the N State to the D State in 6 *M* $GuHCl_6$

Protein	ΔV_t cm³ mol⁻¹ Protein	"Average" residue
Ribonuclease	30 ± 30	0.24
Lysozyme	−30 ± 40	−0.23
Tubulin	0 ± 160	0.00
Chymotrypsinogen A	100 ± 50	0.40
α-Chymotrypsin	150 ± 50	0.62
B.S.A.	750 ± 200	1.27
α-Lactalbumin	40 ± 30	0.31
Lactate dehydrogenase	70 ± 70	0.22
Catalase	240 ± 100	0.44
β-Lactoglobulin	400 ± 60	2.50

From Lee, J. C. and Timasheff, S. N., Partial specific volumes and interactions with solvent components of proteins in guanidine hydrochloride, *Biochemistry*, 13, 257, 1974. With permission.

At the time of writing, the most comprehensive study of pressure folding is that for metmyoglobin[90] with data over a large P-T-pH space. The complexity of the process is evident from Figure 9 which illustrates iso-pH curves in the T-P plane corresponding to the midpoint of the N→D transition, where $\Delta G_{N\rightarrow D} = 0$. It is seen that the

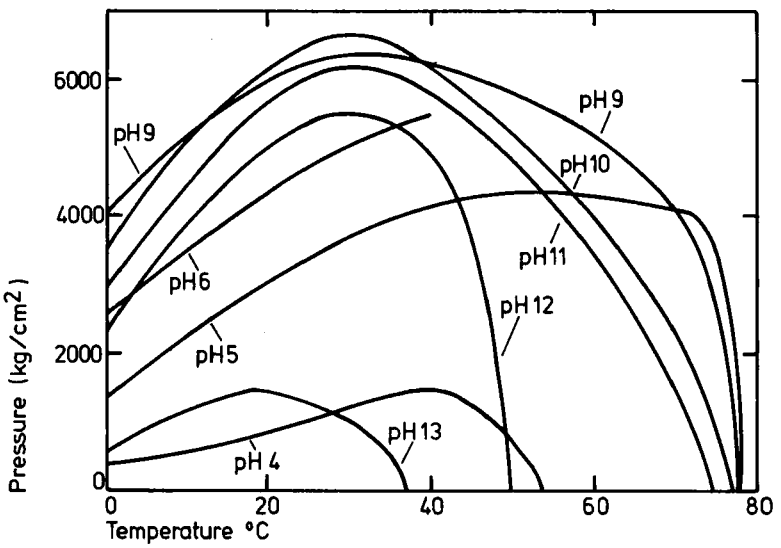


FIGURE 9. Iso-pH curves in the P–T plane for the unfolding of metmyoglobin. Curves correspond to $\Delta G^{\circ}_{N\rightarrow D} = 0$ (within the area enclosed by the contours the N state is stable). (From Zipp, A. and Kauzmann, W., Pressure denaturation of metmyglobin, *Biochemistry*, 12, 4217, 1973. With permission.)

temperature of maximum stability (T_{max}) is pH dependent, but can be high, e.g., at pH 5.6 in the region of $4 - 6 \times 10^3$ kg cm^{-2}, $T_{max} > 50°C$. It is remarkable that for $4 < pH < 13$ there exists a range of pressures where the denatured protein spontaneously refolds and then unfolds again with rising temperature at constant pressure.

ΔV increases from -100 to -60 cm^3 mol^{-1} as the pH increases from 6 to 10. ΔC_p is large but almost insensitive to pressure up to 3,800 kg cm^{-2} beyond which it does decrease to 0 at 5,000 kg cm^{-2}. Such a decrease is compatible with the effect of pressure on hydrophobic interactions, but ΔV should show a much smaller increase with increasing pressure, changing sign at approximately 2,000 kg cm^{-2}.

If both ΔS and ΔC_p vary with pressure, it can be shown that T_{max} should decrease with increasing pressure, but the opposite is found to be the case. It must be concluded that the "simple" hydrophobic group exposure model does not explain the behavior of the protein under pressure, and it must therefore be questioned whether the apparent adequacy of the model at ordinary pressures should be taken for granted.

Equation 3 can provide information about the compressibility of the N and D states. Up to this point we have stressed the classical "oil drop" model[35] for globular proteins, although it has already been noted that the packing of residues is nonuniform.[29]

Studies of the effects of pressure on ribonuclease led Brandts to comment on the extremely low coefficient of compressibility of the protein ($< 5 \times 10^{-6}$ bar^{-1}), comparable to that of solid tin or rock salt rather than a liquid oil drop.[81] Application of the scaled particle theory to the protein interior has provided an estimate of the fraction of filled space within the molecule.[91] For proteins of widely differing molecular weights, this yields an average value of 74.7 ± 0.7% which, when compared with water (36%), cyclohexane (44%), and for cubic packing of identical spheres (~70%), suggests that the protein interior is quite densely packed. Hard sphere models built on the basis of X-ray data[92] also show dense packing of the protein interior and the recent studies[93] of the spectral changes associated with tyrosyl chromophores during the unfolding of ribonuclease suggest that the buried residues are in a "solid-like" rather than a "liquid-like" environment. The data also suggest that those tyrosyl chromophores which are accessible to the solvent reside in an environment which has a more "solid-like" character than normal liquid water.

The hard sphere models also predict that the transfer of a side chain residue from the protein interior to an aqueous environment will be accompanied by relatively small changes in the volume of the system, a situation which is in accord with the experimentally determined ΔV; however ΔV is regarded as being due solely to changes in the volume of the protein and no allowance is made for any contribution from solvent relaxation effects, the existence of which is usually thought to be reflected in the large positive ΔC_p. If this were included, then since the free energy change associated with a volume change ΔV is given by $\int_{\kappa dV}^{\Delta V_{N \to D}}$, the free energy change associated with a ΔV of -45 cm^3 (for ribonuclease unfolding) would be on the order of -900 kJ mol^{-1}. The compensating energy change arising from volume changes in the water, assuming a normal compressibility for water, would only be about 10% of that contributed by the protein and there appears to be no direct indication for such large free energy changes in the unfolding process, in fact, the evidence is very much to the contrary.

On the other hand, current spectroscopic studies of the detailed motions of individual side chains buried in the interior of crystalline globular proteins indicate that a great deal of librational, or even rotational, freedom exists for many side chains. Of course, such findings are hard to reconcile with a rigid, solid-like model of the protein interior.

It is now apparent that explanations of protein denaturation based upon the relatively simple picture of hydrophobic hydration and hydrophobic interactions are inadequate, and if further experimental work confirms the finding that proteins other than ribonuclease also exhibit such a remarkably low compressibility, then we are still far from a satisfactory explanation for protein stability and unfolding.

In addition to the above problems which are mainly thought to be more relevant to globular proteins, fibrous proteins appear to provide their own distinct problems. Collagen, the most common mammalian protein, exists as a triple helix of three polypeptide chains, each of which winds in a left hand sense upon itself, but the combination of the three wind in a right hand sense. The stability of this complex molecule has been related to

interchain hydrogen bonds, but recent data suggest that this cannot be the case. Each collagen polypeptide chain contains approximately 30% gly and 25% pro and HOpro, and it has been reported several times[94,95] that the denaturation temperature, T_m, of the protein increases in a linear manner with the number of gly-pro-OHpro triplets in the molecules. Calorimetric data of the thermal denaturation of several collagens are presented in Table 6: the variation of ΔH with imino acid content (-gly-pro-OHpro) demonstrates the importance of the enthalpy change in the transition, but this is in direct contradiction to the idea that the molecule is stabilized by a network of intramolecular hydrogen bonds, since pro and OHpro are incapable of forming intra-peptide hydrogen bonds. The sign of ΔH appears to suggest that hydrophobic contributions from the pyrrolidine residues are unimportant although laser Raman studies[96,97] have suggested that solvent immobilization occurs around the pyrrolidine rings in the homopeptides poly-L-proline and poly-L-hydroxyproline, and the immobilization of at least some of these water molecules might be expected to occur during the formation of the collagen triple helix. Privalov and Tiktopulo[98] suggest that the stability of the molecular structure arises from the formation of water bridges in the region of the imino acid groups, coupled with several molecular hydration layers adjacent to the protein surface. Several different investigations[99,100] do in fact support the view that collagens are more heavily hydrated than globular proteins but do not provide corroborative evidence for a firmly bound extensive hydration network such as proposed by Privalov and Tiktopulo.[98] The proposal of water bridging within the collagen molecule is supported, at least in part, by computer studies which suggest that half of the bonding between the chains occurs through water molecule bridging and that additional water molecules occur on the periphery of the protein and that these also contribute to the stabilization of the structure.[101]

The importance of electrostatic interactions in maintaining the conformational stability of proteins has been emphasized by a number of authors.[102] They arise mainly from the presence of ionizable groups on the surface of the protein which are capable of titration. If an ionizable group is buried within the protein interior in the native conformation, its pK will differ by several units from its normal value. If such groups revert to normal pK behavior during unfolding, then the degree of proton binding changes, with resulting contributions to $\Delta G_{N \rightarrow D}$.

Electrostatic effects have been studied in rather more detail with solutions of synthetic polyelectrolytes and it has been found that the conformational states so characteristic of proteins also exist, although in a much more rudimentary form, in copolymers of alkyl vinyl ethers and maleic acid.[103] The same factors which influence the protein N→D transition also affect the transition from a hypercoiled to an extended form of the synthetic polymer, and backbone and side chain effects have been postulated.

In addition to specific ionization contributions, it must be remembered that proteins in aqueous solution constitute, in the true sense of the term, a colloidal solution; the disperse particle, i.e., the protein macromolecule, has associated with it a charged atmosphere, or electric double layer, the thickness of which is independent of the nature of the protein and is primarily a function of the concentration and valence of the electrolyte in the dispersion medium. At uni-univalent electrolyte

TABLE 6

Thermodynamic Parameters for the Denaturation of Collagen of Different Imino Acid Contents in Aqueous Solution (pH 3.5)

Collagen	Pro, HOPro/ 1,000 residues	T_m °C	$\Delta H_{N \rightarrow D}$ J mol^{-1}	$\Delta S_{N \rightarrow D}$ J $(mol\ deg)^{-1}$
Rat	226	40.8	6,426	20.6
Pike	199	30.6	5,208	17.2
Merlang	–	21.5	3,696	12.6
Cod	155	20.0	3,150	10.9

Modified from Privalov, P. L. and Tiktopulo, E. I., Thermal conformational transformation of tropocollagen, I. Calorimetric study, *Biopolymers*, 9, 127, 1970.

concentrations of approximately 0.1 m, the double layer thickness is suppressed to <1 nm and becomes of negligible proportions; this results in marked changes in the properties of flexible macromolecules such as proteins.[99,104,105] Some estimate of the contribution to $\Delta G_{N\rightarrow D}$ may be obtained by assuming that the protein molecule is a linear polyelectrolyte and can be modeled by a uniform and continuous line charge; $\Delta G^{dl}_{N\rightarrow D}$, the double layer contribution to the conformational equilibrium, can be written as follows:

$$\Delta G^{dl}_{N \rightarrow D} = \eta kT \ln \kappa\rho,$$

where κ, the reciprocal of the double layer thickness is given by:

$$\kappa^2 = (8\pi n e^2 z^2 / \epsilon\, kT),$$

where n is the ionic concentration in g ion cm^{-3}, z is the ionic valence and, ϵ is the bulk dielectric permittivity; ρ is the axial radius of the protein molecule and η is given by:

$$\eta = \xi_D^{-1} - \xi_N^{-1},$$

ξ being the linear charge density

$$\xi = e^2 / \epsilon\, kTb \qquad (4)$$

where b is the average spacing of the projection of the charged groups onto the axis of the protein. Since the axial thickness of the protein and the thickness of the double layer become approximately equal at an ionic concentration of 0.1 molal the double layer contribution will approach zero at this point, but at lower ionic concentrations this provides a complicating factor which must not be overlooked. It must also be remembered that most common buffering systems contain di- or trivalent ions, in which case ion-specific effects would already become important below 0.1 m. Reference to such ion specific buffer effects is made in Section VI.B.

V. PROTEIN HYDRATION

Many biochemical processes have as one of the initial steps the binding of a small molecule or ion to a macromolecule. Since biological macromolecules only exhibit biological function in aqueous media of fairly narrowly specified ranges of composition and temperature in general, it is likely that native conformations can be described in terms of water–protein interactions, i.e., "water binding", and various other equilibria which determine the nature of the aqueous substrate, e.g., H^+ and OH^- equilibria, and ion and small molecule displacement of water in region A of Figure 6. This raises the vexed problem of a proper description of the system in terms such as bound water, ion binding (e.g., H^+ Ca^{++}), etc. The question also arises how changes produced in the spatial, orientational and dynamic properties of water in region B can affect the observed binding of solvent and small molecular species in region A. All this assumes of course that conformational processes do in fact rely on the binding interactions.

We have already discussed briefly the available experimental techniques and the type of information one might hope to extract from their application. Kuntz and Kauzmann have provided a timely review of what might be taking place in the primary hydration sphere of a protein[6] and this should be compulsory reading for those interested in the intricacies of binding phenomena.

In this discussion we shall only make a brief mention of "permanently bound water", i.e., localized water molecules in crystalline proteins or protein films or fibers held at low relative humidity,* although it must be emphasized that X-ray and neutron diffraction are helping to locate an increasing number of very specifically placed water molecules in crystalline proteins; e.g., 40 such water molecules have been identified and placed in the papain molecule[106] and it is interesting to study their relative positions and orientations relative to the polypeptide. Such a study certainly makes the concepts of an "outside" and an "inside" of the globular protein more blurred. Certain such water molecules or chains of two, three, or more such molecules, hydrogen bonded to strategic backbone or side chain groups, provide an effective pathway for proton exchange, e.g., in carbonic anhydrase. Similarly, 51 water molecules have been identified in chymotrypsin of which 16 are "internal".[107]

Molecules of globular proteins in crystals usually touch one another at several points and create

*There are indications that at least some proteins undergo conformational transitions at low moisture content, so that measurements made under such conditions cannot necessarily be compared with the native protein in solution.

TABLE 7

Hydration of Amino Acid Residues at -35°C

Hydration mol H_2O/mol residue	Amino acid
0	Phe
1	Gly, ile, leu, val, cys, met (ala = 1.5)
2	Trp, ser, thr, gln, asn, asp (pH 4), glu (pH 4)
3	Pro, arg (pH 10), arg^+, tyr
4	HOPro, his^+, [lys^+, lys (pH 10) = 4.5]
6	Asp^-
7.5	Glu^-, tyr^- (pH 12)

From Kuntz, I. D., Hydration of macromolecules. III. Hydration of polypeptides, *J. Am. Chem. Soc.*, 93, 514, 1971. With permission.

irregular voids that remain filled with water. At 2 to 3 Å resolution, the water oxygen atoms are quite indistinct, but it has been suggested that for carboxypeptidase[108] and myoglobin[109] some hundred water molecules are identifiable, but there is no readily apparent "water structure". The molecules seem to serve as proton donors and acceptors for the hydrogen bonding sites on the protein surface. Thus the structure of rubredoxin[110] shows up 105 water molecules on the *surface* of the protein.

The term protein hydration is here used to characterize the protein–water relationship in the *absence* of perturbants which might be regarded as competitive binding or dehydrating agents.* Much of the experimental work has been based on thermodynamic measurements (isopiestic, calorimetric) and therefore suffers from the usual shortcomings of such techniques. These have been succinctly stated by Klotz:

> "Of course from K_i's (binding equilibrium constant) alone one obtains no direct insight to the molecular nature of specific individual binding sites. The thermodynamic analysis in itself cannot reveal molecular features, although it does establish the constraints within which molecular interpretations must operate. Molecular details of the interactions of proteins with small molecules can be disclosed by a variety of spectroscopic probes."[111]

We must add another proviso, namely that interpretations of water activity measurements in terms of adsorption isotherms are of necessity limited by the assumptions and approximations inherent in the mathematical derivation of the isotherm itself. Thus the application of the BET isotherm to the adsorption of water by proteins seems to be a highly questionable exercise to us, irrespective of whether a hypothetical monolayer coverage derived from this treatment agrees well with hydration values obtained by other techniques.

The water implicated in protein hydration has been equated to the "unfreezable" water, i.e., that fraction of water in a solution which does not freeze at or just below 0°C. Differential scanning calorimetry (D.S.C.) and high resolution n.m.r. are the most significant techniques in such investigations. The former is based on the assumption that the normal latent heat of fusion of ice is unchanged in such solutions and that the observed deficit in the "melting" signal is due to "bound water".

Probably the most extensive investigations on polypeptide and protein hydration are those of Kuntz and his colleagues who observed that the proton magnetic resonance (p.m.r.) spectrum of "frozen" protein solutions exhibits residual high resolution components which can be attributed to water molecules perturbed by various functional groups and therefore unable to conform with the very stringent spatial and orientational demands of crystalline ice.[55] By extending their measurements to a series of homo- and copeptides they were able to provide estimates of the hydration of amino acid residues as shown in Table 7.[73] The salient features are:

*It must, however, be emphasized that proteins can hardly be studied in the absence of several other ionic species, some of them quite complex, e.g., phosphate or citrate, so that a reference state of an infinitely dilute solution of protein in water is perhaps not very realistic. Indeed, it has been shown[55] that the extent of protein hydration exhibits a maximum value at ionic concentrations of the order of those often used in buffer systems.

TABLE 8

Protein Hydration (g H_2O/g protein) at −35°C, Measured by ^1H_2O p.m.r. and Calculated from the Amino Acid Hydration Numbers in Table 7; It is Assumed that All Residues are Accessible to the Solvent

Protein	State	Observed	Calculated
Gelatin	N	0.45	0.50
Myoglobin	N	0.415	0.45
Bovine albumin	N	0.40	0.445
Bovine albumin	D (urea)	0.44	0.445
Bovine albumin	D (pH 3)	0.30	0.32
Hemoglobin	N	0.42	0.415
Lysozyme	N	0.34	0.335
Chymotrypsin	N	0.33	0.36

From Kuntz, I. D., Hydration of macromolecules. III. Hydration of polypeptides, *J. Am. Chem. Soc.*, 93, 514, 1971. With permission.

1. Ionized side chains are more heavily hydrated than their undissociated counterparts*; therefore pH changes up to pH 6 should be accompanied by changes in protein hydration.

2. Hydrophobic residues exhibit a minimal degree of hydration, therefore exposure of the hydrophobic protein core should result in a very slight increase of total hydration.

3. Considering its molecular structure, proline (and poly-L-proline) exhibits a remarkably high degree of hydration.

4. The width of the p.m.r. signal, but not the degree of hydration, is sensitive to conformational effects in polypeptides; thus α-helices do not trap excess water of hydration.

5. The extent of hydration of a protein is, to a first approximation, the sum of the hydrations of the constituent amino acid residues. This is demonstrated in Table 8.

Further studies[113] on polypeptides have shown that the detailed features of the unfrozen water p.m.r. signal do in some way depend on the conformational properties of the polymer. Thus a signal narrowing is observed with increasing concentrations of NaCl, KCl, or $(NH_4)_2SO_4$ up to 0.1 mol dm^{-3}, beyond which concentration the signal begins to broaden again. Also, the width of the signal appears to be proportional to the degree of internal structure, so that poly-L-valine, which is thought to exist as random coil, exhibits a very narrow water signal, whereas poly-L-alanine, poly-L-proline, and poly-L-glycine show water signal broadening, suggesting nonrandom structures in solution. However, the effects of electrolytes, pH, temperature, and rate of temperature quenching are too complex for simple relationships between amino acid composition, hydration, and conformations of proteins to be established by this method.

An interesting extension of the "frozen solution" p.m.r. studies[114] has indicated that for electrolyte solutions the signal intensity, I(T) decreases with decreasing temperature and disappears at the eutectic temperature. It was also found that only amino acids containing hydroxyl groups exhibit an "unfrozen water" signal and here again I(T) reaches zero at some definite temperature. Furthermore, this temperature is the same for a given amino acid and its homopolypeptide. Rather different results were obtained for proteins, in that I(T) remains constant down to −100°C, but line broadening takes place. From studies of ^1H and ^{19}F resonances on solutions of bovine serum albumin in aqueous KF it was found that while the salt had no effect on the ^1H spectrum, the ^{19}F signal was also narrow, with I(T) independent of the fluoride concentration and proportional to the BSA concentration at a given temperature. Thus the concentration of nonfrozen water is independent of temperature, whereas the concentration of "non-frozen" KF, i.e., its solubility *s*, decreases with decreasing temperature. Also a $\ln s$ vs. $^1/T$ plot is linear to well below the KF/H_2O eutectic at −21.8°C. The implications of these results are important: the "liquid" which exists at subzero temperatures has been identified as water of hydration. Nevertheless, it has the normal solvent properties of water, in spite of the high protein concentration in the system.

By and large the extents of hydration calculated from D.S.C. and n.m.r. agree well. Normally data derived by the former technique are presented as $C_p(T)$. At low water contents, e.g., <30 g/g protein, in the case of tropocollagen,[115] no absorption of heat is apparent at 0°C and only at 46.5 g water/g protein is the normal melting behavior of ice observed. On the basis of a gly-pro-HOpro-model for tropocollagen, this corresponds to 2.4 mol H_2O per residue, in very good

*The opposite conclusion, arrived at by Glasel[112] from a consideration of deuteron magnetic relaxation rates, was probably due to some unwarranted assumptions.

agreement with the value 2.7 obtained from p.m.r. measurements.[113] The same degree of correspondence was also achieved with several globular proteins and the results can be accounted for in terms of a model of the protein in which only polar groups on the exterior surface are hydrated.[116]

Although infrared spectroscopy is particularly informative in the study of biopolymer hydration at low water content,[44,117] nevertheless certain results may hopefully be transferred to describe the hydration in aqueous solution. The peptide bond appears to be the major site of hydration[45] and the application of near infrared difference spectroscopy has enabled McCabe and Fisher[116] to distinguish between the absorption due to water excluded by a hydrated protein, the water affected by hydration interactions, and the protein itself. Similar experiments on the simpler molecules of polyglutamic acid and polylysine have shown up spectral changes corresponding to the α-helical and coiled conformations.[46] Since the transition was brought about by acid titration, the observed changes may reflect the ionization of the ϵ-NH_2 group. In this context it is of interest to note that there is general agreement that $-NH_2$ groups are much more heavily hydrated than NH_3^+ groups.

The foregoing discussion illustrates that the strengths of infrared (and Raman) spectroscopy lie in the identification of macromolecular water binding sites and characterization of different hydrogen bonding environments, but the spectra of water are so complex that a normal mode analysis will be out of the question for some time to come.

On a molecularly less detailed level, but also of considerable interest, is a recent attempt to estimate the solvation contribution to the observed CD spectra of polypeptides and proteins.[118] The assumption is made that the amide groups are the solvation sites and for a dipeptide it is shown how two solvation regions, one on each side of the amide plane, can affect the asymmetry of the peptide, and how the solvent contribution to the asymmetry depends on the conformation of the peptide. By allowing for solvation in the peptide conformational energy map, the author concludes that solvation effects might contribute to the CD spectra of the α-helix, poly-L-proline II, and collagen, but not those of poly-L-proline I or β-sheets.

Another set of methods relevant to the problems of solvent interactions lies on a characterization of the diffusional behavior, both rotational and translational, of the various components.

As regards the protein, the motions of the macromolecule through a viscous continuum must be related to its shape and hydrodynamic volume, the latter including water which has been transported on a time scale of the experiment. It is this factor, expressed in terms of the partial specific volume in sedimentation measurements, which is at the root of much uncertainty. For an up to date critique of the various methods employed in the measurement of rotational diffusion constants of proteins the reader is referred to Reference 6.

A further insight into the dynamic behavior of the system is obtained from a monitoring of the diffusional motions of water molecules. The most effective methods depend on dielectric and nuclear magnetic relaxation techniques which have recently been reviewed by Cooke and Kuntz.[119] Roughly speaking, three "types" of water molecules can be distinguished by their dynamic properties, e.g., the rotational diffusion time, τ_r, defined as the time required to rotate through one radian or diffuse through one molecular length:

1. "Bulk" water whose rotational and translational motions are only marginally affected. Normally $\tau_r = 10^{-11}$ sec, but in the presence of most solutes, even at low concentrations, longer τ_r values are observed. It is therefore an open question whether, and how much, completely unperturbed water exists in a protein solution. In any case this species of water would be found on the outer periphery of region B in Figure 6.
2. A species characterized by rather slower diffusion, typically $\tau_r = 10^{-9}$ sec at room temperature. The motions of such molecules are also found to exhibit anisotropy and are clearly affected by their proximity to the macromolecule, although the dominant interactions are still of the water–water type.
3. Irrotationally bound water, $\tau_r = 10^{-5} - 10^{-7}$ sec, the motions of which are determined mainly by those of the macromolecule.

It must be emphasized that the situation is not nearly as clear cut as the above classification implies. The most important single complicating problem is the correct interpretation of experimentally determined nuclear magnetic and dielec-

tric relaxation rates and their dependence on temperature, frequency and composition in terms of molecular dynamic properties. On the molecular side the complications arise from a multiplicity of binding sites, exchange of water protons or water molecules between different environments, anisotropic diffusion in restricted spaces, and general complexities associated with the hydrogen bonding regime in liquid water. It is well beyond the scope of this review to appraise the different approaches to such problems, but these are topics currently of lively interest, and we can look forward to significant advances. For state-of-the-art reviews the reader is referred to References 6, 106, and 119.

Protein hydration can be described in thermodynamic, structural, and dynamic terms, but there is no quantitative relationship between hydrodynamic and thermodynamic approaches to water binding. In addition the former approach is very model sensitive, and difficulties arise in the specification of solvated particle boundaries. Thermodynamic criteria, such as water activity (a_w), can be applied to distinguish between bound and free (or trapped) water, although their application becomes increasingly difficult as the pockets of trapped water decrease in size. Such water-filled cavities certainly do exist in crystalline proteins.[120] Other shortcomings of the thermodynamic experiments are that they have usually been performed on partially dried proteins (low a_w) or at subzero temperatures on "frozen" solutions. Kuntz and Kauzmann point out that there is no reason to expect that the low temperature measurements have any direct relevance for room temperature hydration.[6]

The same can be said for estimates of internal structural hydration, as detected, e.g., by X-ray diffraction. It also appears that up to 500 water molecules can, in favorable situations, be identified on the external surface of proteins, but as yet we know little about their degree of localization or their role in stabilizing the secondary and/or tertiary structures.

In summary, Table 9 provides a comparison of hydration data obtained by a variety of experimental techniques and interpretations.[6] Kuntz and Kauzmann conclude that the extent of agreement is "mildly encouraging", and this seems to us a generous assessment of the current situation.

VI. SOLVENT EFFECTS ON PROTEIN CONFORMATION IN MULTICOMPONENT SYSTEMS

A. Thermodynamic Characterization of Relative and Preferential Binding

As has been pointed out several times in this review, thermodynamic methods provide a useful means of specifying "binding" in the macroscopic

TABLE 9

A Comparison of Protein Hydration Data Obtained by Different Techniques

		Hydration mol H_2O/mol protein				
Protein	Mol. wt.	P.m.r. (-35° C)	Scanning calorimetry (nonfreezable water)	Hydrodynamic	Isopiestic (a_w = 0.92)	Structural (X-ray)
Ribonuclease	13,700	–	–	434	266	–
Lysozyme	14,300	270	238	365	199	–
Myoglobin	18,000	240	–	460	320	110
β-Lactoglobulin	36,700	–	1,121	918	612	–
Ovalbumin	45,000	825	–	375	750	–
B.S.A.	68,000	1,511	1,208, 1,851	1,624	1,208	–
Hemoglobin	64,500	1,504	1,147	1,792	1,326	–
Subtilisn	27,300	–	–	–	–	273
Chymotrypsinogen	25,700	485	–	543	414	14,* 100

*This value is for the "internal" localized water.

Adapted from Kuntz, I. D. and Kauzmann, W., Hydration of proteins and polypeptides, *Adv. Protein Chem.*, 28, 239, 1974.

sense, i.e., in terms of changes in free energy and enthalpy. They are particularly useful in comparative binding studies. Isopiestic, calorimetric, densimetric, and optical methods have been employed in investigations of relative and preferential binding of solvent components to proteins. In addition, where stoichiometric or specific site binding can be established, pseudothermodynamic methods based on spectroscopy and optical activity can yield binding constants but cannot provide direct information about changes in hydration.

As regards the evaluation of the differential binding of water and other solvent components, several variations on the same basic thermodynamic theory have been published. For the typical isopiestic experiment[121]

$$\frac{n_3}{n_1} = \frac{n_3'}{n_1'},$$

where n_1 and n_3 are the number of moles of water and cosolute in the sample, and n_1' and n_3' are the number of moles in the reference mixture. In the presence of protein, component 2, Δn_1 and Δn_3 moles of water and cosolute are bound so that

$$\frac{n_3 - \Delta n_3}{n_1 - \Delta n_1} = \frac{n_3'}{n_1'}. \quad (5)$$

In practice, Equation 5 requires several corrections, but continuing with its simple form, if Δn_1 and Δn_3 are constant, then

$$n_1 m_3' - C n_3 = m_3' \Delta n_1 - C \Delta n_3 \quad (6)$$

where m_3' is now the *molality* of the reference solution, C = 55.51 and the other quantities are expressed in moles per unit quantity of protein.

Equation 6 can be rearranged to give

$$n_1 = \left[\Delta n_1 - \frac{C \Delta n_3}{m_3'}\right] + \frac{C \Delta n_3}{m_3'}. \quad (7)$$

Although isopiestic methods have been shown to be of great value in protein binding studies, they are limited to systems in which there is only *one* volatile component, i.e., water. More generally applicable thermodynamic methods of estimating *relative* solvent binding in binary solvent systems have been discussed by Timasheff and his associates[122-124]* who then proceed to derive "absolute" values of solvent binding. Once again the solvent species have to be operationally classified as *free* or *bound* with all the weaknesses implicit in such a classification.

The "absolute" amount of organic cosolvent bound to the protein is given by

$$A_3 = (\partial w_3/\partial w_2)_{T,\mu_1,\mu_3} + w_3 A_1 \quad (8)$$

where A_3 and A_1 are here expressed in g/g protein rather than on a molar basis. Hence w_3 and w_2 are now also expressed in grams and μ_i are the chemical potentials. A_1 in Equation 8 is the degree of hydration of the protein. If it were possible to obtain reliable estimates of A_1 and any dependence of A_1 on cosolvent concentration, then it would be possible to calculate the absolute binding of volatile organic species and to assess their influence on protein stability. However, as was shown in the previous section (see Table 9), experimental estimates of A_1 are not too reliable and depend to some extent on the experimental techniques and conditions used. To our knowledge no data exist on the effects of third components on A_1, so that experimental determinations of $(\partial w_3/\partial w_2)$ must suffer from considerable uncertainties unless fairly strong evidence exists for a simple relationship between A_1 and the other solvent component. We return to this problem in Section VI.E.

There are various experimental approaches to the measurement of $(\partial w_3/\partial w_2)$ apart from the isopiestic equilibrium method already described. They include differential refractometry, light scattering, the variation of equilibrium constant of a reaction with change in solvent composition (e.g., the N⇌D equilibrium), and partial specific volume measurements. In the latter case, for instance, A_3 is given by[126]

$$A_3 = [(\partial\rho/\partial w_2)_{T,\mu_1,\mu_3} - (\partial\rho/\partial w_2)_{T,P,m_3}]/(\partial\rho/\partial w_3)_{T,P,m_2}$$

where ρ is the density of the solution which in turn is related to the partial specific volume in the limit of infinite dilution (of protein). If changes in A_3 also produce a conformational transition which

*A very useful summary of the application of the various techniques to the system water–lysozyme–2-chloroethanol is given in Reference 125. The general findings are discussed below in Section VI.F.

is accompanied by a volume change, ΔV, then volumetric measurements allow the determination of A_3 and ΔV.[89]

Somewhat more detailed descriptions of binding processes are based on the mass action law; two alternative methods are available.[111] The first is based on the assumption that binding is stoichiometric and that a series of stoichiometric equilibrium constants can be fitted to the experimentally determined number of moles of solute bound. The other approach assumes the existence of binding sites with different affinities, and hence independent site equilibrium constants can be defined and once again the total number of ligand molecules calculated. It must be remembered that both these treatments are essentially just curve fitting procedures, unless independent, nonthermodynamic evidence is available as to the number and nonequivalence of sites. Also no information can be derived about the binding of solvent or its displacement by other ligand species. The methods based on mass action binding constants are particularly useful where a limited number of sites exist, e.g., it has been shown quite clearly that only two moles of leucine are bound by α-isopropylmalate synthetase,[127] and all the quantities associated with this binding equilibrium are compatible with this estimate.

Finally before leaving the subject of the general thermodynamics of solvent component binding by proteins, it may need emphasizing that in all the above equations, concentrations should be replaced by activities. Unfortunately almost no activity coefficient data exist and therefore the assumption is always made that in dilute solutions activities can be adequately approximated by concentrations. Three cautionary comments are in order:

1. In the vicinity of a highly charged species such as a protein molecule, this assumption may not be valid.

2. Experimental estimates of $(\partial w_3/\partial w_2)$ indicate that again, in the vicinity of the protein, high concentrations of cosolvent can exist, i.e., the condition of the dilute solution is not met.

3. Aqueous solutions of certain organic compounds – notably the hydrophobic species – exhibit very complex activity/concentration behavior especially in dilute solution and at low temperatures.[20,128]

B. Ion Binding to Proteins

A change in the hydration environment of a protein by means of ions can be achieved in two ways: by the addition of neutral electrolyte, or by a change in pH which leads to changes in the dissociation equilibria of acidic and basic residues. Either or both of these methods may promote the isothermal N→D transition. On the other hand, a given electrolyte concentration may not disrupt the N state at room temperature, but it will affect the thermally induced N→D transition by lowering or raising the transition temperature T_m. Under certain conditions the N state may be induced to undergo a somewhat different conformational change, to a more ordered state, which in its organization resembles the N rather than the D state, and here is called the E (for extended) state.

Let us deal first with the hydration/ion binding where no gross conformational transition occurs. It has been pointed out by several authors[104] that at low electrolyte concentrations, ionic strength $I \lesssim 0.1$ mol dm^{-3}, no ion specific effects are observed; in fact, the solubility and general electrochemical behavior of proteins are proportional to I. This seems to be a general result which applied equally well to other aqueous polyelectrolytes and to hydrophobic colloids. It can therefore be argued that at $I<0.1$, the main function of the ions in solution, is the modification of the nature and thickness of the electrical double layer surrounding the protein molecule, as for instance described by Equation 4.

At higher ionic concentrations the ion-specific effects characteristic of the Hofmeister series[17] become dominant. In view of the well-authenticated nature of these ion-specific effects in a large number of biochemical processes, it is surprising that in the majority of biochemical publications salt effects are still treated indiscriminately and expressed in terms of the ionic strength.[105] Sometimes even the buffer components, almost invariably present in protein systems, might be expected to produce major changes in the solvent environment of a biopolymer.

Unfortunately investigations of the effects of low salt concentrations on protein conformational stability are rare, but Ginsburg and Carroll have reported the influence of different buffer systems at low ionic strength (0.019) on T_m of ribonuclease.[129] Relative to a HCl/KCl buffer, $H_2PO_4^-$/H_3PO_4 produced a ΔT_m of 12° and SO_4^{--}/HSO_4^- of 14°. The width of the N→D transition was also

shown to be significantly narrowed in the sulfate buffer, indicating an increased cooperativity. Thus for the HCl/KCl system, $\Delta H_{vH} \simeq 200$ kJ mol^{-1}, whereas in SO_4''/HSO_4' the corresponding value is $\simeq 300$ kJ mol^{-1}. These and other studies highlight once again the specific effects which govern the conformational processes. It is therefore hardly helpful to express electrolyte effects in terms of pH and ionic strength.

The careful isopiestic studies of Bull and Breese,[121] although limited to high concentrations, provide informative data on the apparent effects of different electrolytes on protein hydration in terms of Equations 6 and 7. Figure 10 represents the binding of alkali metal chlorides to egg albumin. Also indicated are values for Δn_1 and Δn_3 for the various systems, calculated from the linear portions of the curves by use of Equation 6.

For the one system (CsCl) where a comparison can be made with other independent results,[130] the agreement is reasonable. The Δn_1 results in Figure 10 demonstrate that the cation Hofmeister series is operative in reducing the degree of protein hydration-in the absence of added electrolyte, egg albumin binds 740 mol water/mol.*[131]

It is interesting to note that neither NaCl nor KCl show any very pronounced binding,** and that the alkali metal chlorides (with the exception of NaCl) exert their main effect as dehydrating agents. The opposite effect is observed in the presence of Na_2SO_4, and to a lesser extent NaBr. Thus, if it is assumed that $\Delta n_3 = 0$ for Na_2SO_4, then the estimated $\Delta n_1 = 1350$! The opposite effect is observed for NaI, NaCNS, and $CaCl_2$, all of which are preferentially bound to the protein at the expense of water. This almost certainly accounts for their protein denaturant effects.

The situation in the complex mixtures under discussion is not quite so simple as the straightforward estimates of Δn_1 and Δn_3 might imply. In the first place, Δn_3 should be separated into cationic and anionic contributions. Also the ions are not likely to interact just with the water in the inner (A) protein hydration sphere, but with water in bulk, however, this may be defined in a protein solution. Thus Bull and Breese[131] comment pertinently that at isopiestic equilibrium (in the absence of protein) the alkali halides coexist in the following concentrations: LiCl – 1.78 m, NaCl – 2.00 m, RbCl – 2.18 m, CsCl – 2.28 m which reminds us once again of Hofmeister's speculation already referred to.

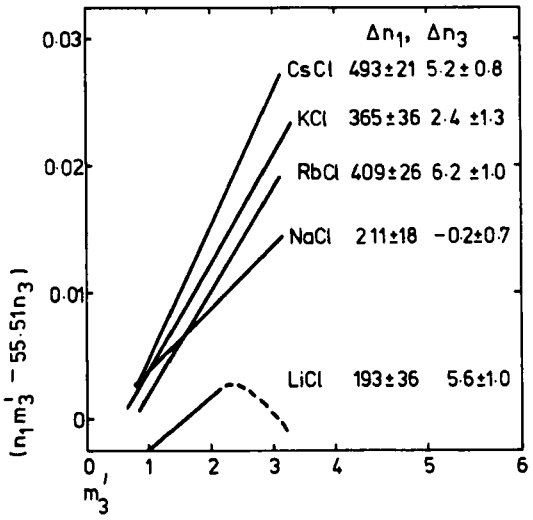


FIGURE 10. Isopiestic ion binding data for egg albumin (see Equation 6),[121] measured at $a_w = 0.92$ and 25°C. NaCl and KCl show little binding, and the main effect of all salts is a reduction in Δn_1 which, in the absence of salt, has a value of 740 mol/mol.

Ion binding, as described in the above paragraphs, is unlikely to occur without at least some minor changes in the conformation of the protein, but more dramatic electrolyte effects are reflected in the denaturation and possibly the subsequent association and precipitation of proteins.

C. Ion Induced Conformational Transitions in Proteins

We have so far treated protein hydration and ion binding as processes which do not dramatically perturb the conformational stability of the protein, i.e., the discussion has been limited to changes on the periphery of the N state. This is not to imply that minor conformational changes do not occur as a result of ion binding. In the present section the main concern is the destabilization of the N state brought about by ions. There is a large volume of experimental data concerning the effects of electrolytes on the thermally induced N→D transition, and for an introduction to this subject the reader is referred to the concise

*Perhaps this result should be treated with some caution as it refers to experimental conditions of 92% relative humidity.
**In view of this it is perhaps not coincidental that Na^+ and K^+ ions play such an essential role in physiological processes.

and authoritative review by von Hippel and Schleich.*[132]

We shall now confine ourselves to a summary of the salient features of the processes. One of the most significant findings has been that various types of thermodynamic equilibria respond in a similar manner to ionic perturbations. They include:

1. N→D transitions of globular proteins
2. α-helix→coil transitions of homopolypeptides
3. Double helix→coil transitions of polynucleotides and polysaccharides
4. Triple helix→coil transition of collagen to gelatin
5. Monomer ⇌ micelle equilibria of surfactants
6. Salting in and out from aqueous solution, and hypercoil→extended form transitions of synthetic polymers

but this is by no means an exhaustive list.

The effect of salts on the transition temperature of any of the above equilibria can be expressed by:

$$\Delta T_m = T_m - T_m^\circ = k_{ij}c_{ij} \qquad (9)$$

where T_m and T_m° are the transition temperatures in the presence and absence of ionic species i and j at molar concentration c_{ij}. It is seen that Equation 9 predicts a linear relationship between ΔT_m and c_{ij}, with k_{ij} as the molar shift in T_m. Figure 11 shows the effect of electrolytes on the N→D transition of ribonuclease which shows that Equation 9 provides a fair approximation to the actual behavior, at least at low c_{ij}. By combining results for series of salts with common ions, k_{ij} can be divided to yield ionic contributions k_i and k_j to ΔT_m.

The mechanism by which the ions exert their effects on macromolecular conformation has often been related to their solvent power, i.e., the way in which they influence the solubility of the various chemical groupings which are normally in the "interior" of a folded molecule but become exposed during the unfolding process. Hence, the ionic effects on the N→D transition are related to

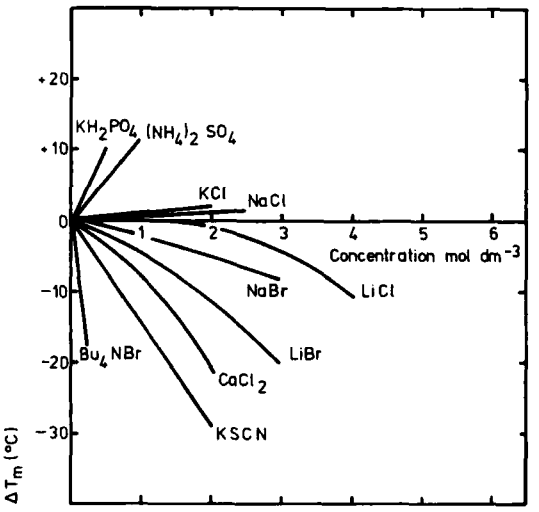


FIGURE 11. ΔT_m produced by different electrolytes on the N→D transition of ribonuclease (at pH 7 and in the presence of 0.15 *M* KCl and 0.013 *M* sodium cacodylate). (From von Hippel, P.H. and Wong, K.Y., The effects of various electrolytes and nonelectrolytes on the thermal ribonuclease transition, *J. Biol. Chem.*, 240, 3909, 1965. With permission.)

salting-in or salting-out effects observed with quite simple molecules, e.g., benzene, which also obey a relationship of the type of Equation 9, known as the Setschenow equation:

$$\log \gamma = \log (s^o/s_{ij}) = k'_{ij}c_{ij}, \qquad (10)$$

which relates the activity coefficient γ of the nonelectrolyte species to its solubility in water (s^o) and in electrolyte (s_{ij}) and hence to the electrolyte concentration c_{ij}, where k'_{ij} is termed the Setschenow or salting-out coefficient. Supporting evidence for the small molecule solubility analogy is provided by the same apparent additivity of ionic effects on ΔT_m and log (s^o/s), although such additivity is difficult to rationalize.

However helpful such a qualitative comparison may be, there are marked quantitative differences between ionic effects on solubility and ΔT_m. Thus NaCl and KCl are effective salting-out agents for benzene but have almost no effect on ΔT_m (see Figure 11).

The salting-in/out mechanism is based on the postulate that a globular protein is composed of two types of chemical groupings: polar (backbone and side chain) and nonpolar (side chain). In the N

*See also von Hippel, P. H. and Schleich, T., in *Biological Macromolecules*, Vol. II, Timasheff, S. and Fasman, G., Eds., Marcel Dekker, New York, 1969, p. 417 for a more detailed account.

state the polar groups are exposed to the solvent, whereas the apolar residues are in the interior of the molecule.* Model experiments and the separation of k_{ij} into contributions due to polar (peptide) and apolar side chains led Schrier and Schrier[133] to the conclusion that the *specific* ion effects arise from the salting-in of apolar groups, whereas the exposure of additional peptide or other polar groups contributes a constant salting-in contribution. This conclusion is not easily reconciled with von Hippel's more recent ion binding experiments, already referred to in Section III.C.

Recent experiments on model peptides and amide derivatives have yielded k'_i and k'_j values in Equation 10.[134-136] The most effective protein denaturants have been identified as the most potent salting-in agents for peptide groups. By a set of similar experiments but employing the same model compounds so modified that each peptide unit also carried a nonpolar residue, and then subtracting the previously determined salt effects, the authors were able to obtain the nonpolar side chain contributions to the salt effects. They established that the ions most effective at salting-in the peptide were the least effective in salting-out the nonpolar residues, but the SO_4'' ion which tends to salt-out peptide groups has a strong salting-out effect on apolar side chains, hence its remarkable stabilizing effect on the N state. Once again the relative order of ionic effects corresponds closely to the Hofmeister series, both for cations and anions. Thus in addition to the additivities in k_i and k_j, a second class of additive behavior appears to operate with respect to polar and nonpolar groups.

Various attempts have been made to rationalize the observed salt effects on ΔT_m in terms of the electrostrictive effect of ions on the internal pressure of water,**[137] or in terms of the scaled particle theory[138] (see Section III.C). None of these treatments is really satisfactory and arbitrary and/or unpalatable factors must be introduced to obtain reasonable agreement with the experimental results. One of the problems is of course that in a protein each apolar group is located in the immediate vicinity of one or more polar groups and the elegant experiments of von Hippel have shown the subtle nature of the mutual interactions between amide dipoles, vicinal methyl groups, and hydrated ions.

Another problem of the "simple" salting-in/out model is associated with the behavior of linear polymers such as fibrous proteins, nucleotides, polysaccharides, and those synthetic polypeptides which exhibit N→D transitions. The molecular structure of all these polymers is such that side chains and, presumably, polar backbone groups are exposed to the solvent in both the N and D states, and yet the salt effects on ΔT_m follow closely those described for globular proteins.

Some of the unresolved questions can be summarized as follows:

1. How is $\Delta G_t^{\ominus}$ of various macromolecular groupings, i.e., transfer from an apolar to an aqueous environment, affected by ions? Are there specific hydrophobic or other hydration states which interfere destructively with the ion-dipole interaction?
2. Is the estimation of water and ion "binding" likely to throw further light on the nature of the N→D transition? How is binding, as determined isopiestically, related to salting-in or out?
3. Since ionic effects appear to be additive, does this mean that "binding", as revealed by isopiestic experiments, is irrelevant or is there a multiplicity of ion specific sites?
4. If cations as well as anions can interact with the amide dipole, can such interactions alter the common transplanar conformation of the peptide bond, and hence the tertiary structure of the macromolecule?

Finally, to describe the state-of-the-art and bearing in mind that water is the only common factor in all the systems under discussion, we once again quote from a previous review:[132]

"....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 3-component problem, and an analysis of the interaction of any two components will provide information which is relevant

*Since each leu, val, ileu, phe, etc. group is attached to a polar backbone group, these latter must presumably also be situated in the interior of the folded protein.

**The use of internal pressure can lead to difficulties with water at low temperatures, where the coefficient of expansion changes sign at 4°C.

and necessary, but not sufficient, to the solution of the problem."

D. Chaotropism Induced by Urea and Guanidinium Salts

We have seen that the effects of ions on the conformational stability and transitions of proteins exhibit specificities above certain limiting concentrations (~0.1 m for uni-univalent electrolytes) and that these specificities are related to the Hofmeister series which in turn is believed to arise from sensitively balanced ionic, dipolar, and nonpolar solvation effects.

There is another class of destabilizing (or chaotropic) agents which, we believe, act by a somewhat different mechanism, and evidence is accumulating which suggests that the D conformations so produced differ from those produced by ions (and by other organic destabilizers – see section VI.E).

It has long been known that both urea (U) and guanidinium (GuH^+) salts are very effective protein denaturants. In the case of GuH^+ salts, the situation is complicated by the anion contributions to the observed unfolding equilibria. Commonly GuHCl is used in studies of N→D transitions, but the complicating anion effects are strikingly illustrated in Figure 12 for the thermal unfolding of ribonuclease.[139] It is seen that SO_4'' ions more than offset the ΔT_m due to GuH^+ so that the net effect is one of stabilizing the N conformation of ribonuclease. Such competing effects have also been observed with urea denatured ribonuclease, thus phosphates (in particular cytidine 2′-phosphate) can accomplish the refolding of the protein in 8 *M* urea.[140] In the following discussion we shall be concerned with the chaotropic effect of the GuH^+ ion, although most of the experimental evidence cited will be based on GuHCl; it is assumed therefore that Cl' ions have a minimal effect on the N→D transition.

One important clue to the difference between U and GuH^+ on the one hand and ions or other organic compounds on the other becomes apparent from a study of Figure 13 which summarizes $\Delta G_t^{\oplus}$ data for peptide groups and leucyl side chains. In general, as expected, electrolytes salt-in peptide groups and salt-out apolar residues, and the opposite effects are observed for organic solvent additives, such as dioxan and ethanol, which are known to lower the dielectric permittivity of water. U and GuH^+ are unique in that

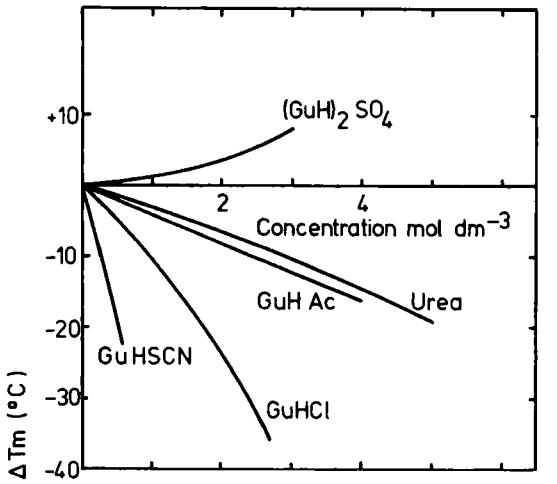


FIGURE 12. ΔT_m produced by urea and different GuH^+ salts on the N→D transition of ribonuclease (conditions as in Figure 11). (From von Hippel, P. H. and Wong, K.Y., The effects of various electrolytes and nonelectrolytes on the thermal ribonuclease transition, *J. Biol. Chem.*, 240, 3909, 1965. With permission.)

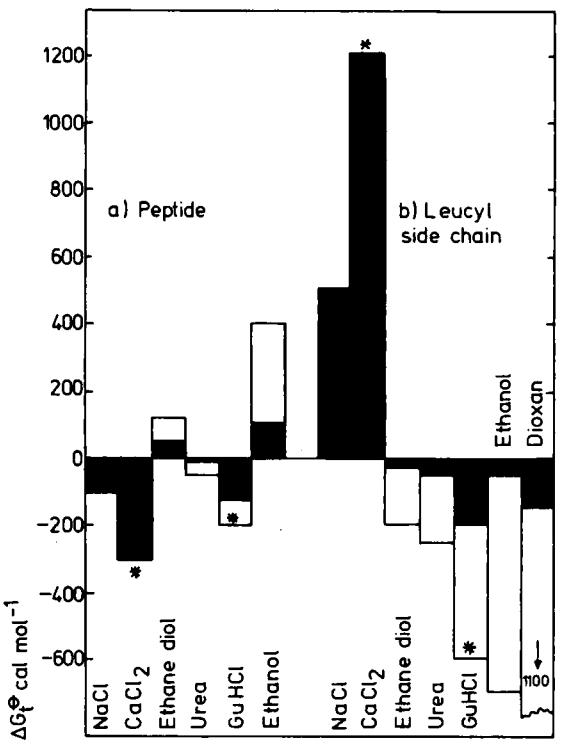


FIGURE 13. $\Delta G_t^{\oplus}$ of (a) peptide groups and (b) leucyl side chains corresponding to a transfer from water to 2 *M* (dark areas) and 8 *M* (light areas) aqueous media. *Refer to extrapolated values. (From Franks, F., The hydrophobic interaction, in *Water – A Comprehensive Treatise*, Vol. 4, Franks, F., Ed., Plenum Press, New York, 1975, chap. 1. With permission.)

they increase the solubility of dipolar peptide groups *as well as* leucyl side chains.* This observation alone can account for their potency as destroyers of noncovalent macromolecular structures.

However, these findings raise further questions about the mechanism or mechanisms whereby such universal salting-in** effects can arise, what molecular features are responsible, and how the observed model compound $\Delta G_t^{\ominus}$ studies relate to the manner in which U and GuH^+ promote N → D transitions of a wide variety of chemically quite dissimilar aqueous polymers. Table 10 shows that the subject of U and GuH^+ facilitated unfolding of proteins is certainly of current interest. The table lists some (but not all) recent significant contributions in this general area; the diversity of the conclusions reached by different workers as to the nature of the interaction (if any) between U or GuH^+ and proteins suggests that this problem still awaits resolution. As is so frequently the case in the discipline of Biochemistry, it is hard to compare results derived from different investigations because of the variety of biochemical systems, experimental conditions, and measuring techniques; any comparisons must therefore contain a large component of speculation. All we can do, therefore, is to survey critically the available information and emphasize areas of concurrence and contradiction and finally arrive at what seems to us the most likely mechanism of action of U and GuH^+.

We start by assuming that the two chaotropic species under discussion act in a similar manner qualitatively (although probably not quantitatively). Since the common factor in all the systems is the water plus denaturant mixture, it is pertinent to review the state of knowledge concerning aqueous solutions of urea.[143] The balance of the available evidence[144] suggests that:

1. U destroys the three-dimensional hydrogen bonded order, characteristic of water at ordinary temperatures.

2. No alternative long-range order can be observed even at urea concentrations as high as 10 m, where the mole ratio U:water is 1:5.5.

3. The destruction of long-range order in water is not due to strong solvent-solute interactions, such as preferential urea-water hydrogen bonds, but is more akin to a statistical effect in which all hydrogen bonding interactions are equally probable, but the planar geometry of U precludes the tetrahedral coordination, characteristic of water.

4. Even in very concentrated urea solutions the rapid rotational motions of U preclude the existence of long-lived urea polymers.

The above factors must be kept in mind when the influence of U and GuH^+ on protein conformation (in water) are considered. Reference to Table 10 shows that there exists an unacceptably large amount of disagreement in the interpretation of what must certainly be reliable experimental results. Apart from some general understanding of the aqueous denaturant solutions, information is required about the following aspects of the ternary systems water–protein–denaturant:

1. The nature of the D state and the possible existence of intermediate, partly unfolded states.
2. The degree of binding of U or GuH^+ and the nature and location of the binding sites.
3. Relative binding affinities of the N and D states.
4. The effect of denaturant binding on the degree of hydration of proteins.

Opinions about the D state vary: Tanford[58,145] has suggested that the properties of both ribonuclease and lysozyme are compatible with those of a random coil in 6 *M* GuHCl, since the pH titration curves agree closely with the predicted curves based on the pK_a data for the constituent amino acids; all residues are therefore accessible to the solvent. Supporting evidence for a random coil D state comes from calorimetric unfolding studies.[146] It is an open question whether the pH

*That U can "salt-in" (enhance the solubility) of "real" apolar compounds, such as alkanes is well established[141] and the possible origin of such processes has been studied[142] in terms of the peculiar nature of aqueous urea solutions referred to below.

**To use the term salting-in, when referring to a nonelectrolyte, is of course incorrect, perhaps we should refer to the phenomenon as hydrotropism or solubilization. However, "salting-in" is used so universally in the literature that we shall continue to describe the effect in this manner, bearing in mind, however, that its origin cannot be of an electrostatic nature.

TABLE 10

Summary of Recent Experimental Studies of N → D Transitions Induced by Urea and Guanidinium Salts

Reference	Date	System	Experimental technique	Main conclusions
148	1967	Ribonuclease	UV absorption	Average conformations of N and D states are similar, therefore, the large ΔC_p is due to the exposure of apolar groups; but ΔC_p is almost independent of solvent composition. At high U concentration, the protein undergoes an inverted transition and the concentration of N reaches a maximum at a characteristic temperature. N.B.: this temperature of maximum stability is also found in the absence of U.
58	1967	Ribonuclease	pH titration	In 6 *M* GuHCl the protein is a random coil, even with intact S–S bonds. The titration curve is predictable from known pK_a values of the constituent amino acids.
150	1969	Myoglobin	UV absorption and optical rotation	Conformations of several D states vary with the kind and concentration of denaturant and only approximate a random coil at very high U concentration. Secondary structure is maintained and may resemble that of the N state.
85	1969	Lysozyme		Strong binding of GuH^+ to aromatic side chains and pairs of adjacent peptide groups. Either more GuH^+ is bound to D form *or* more water bound to the N form.
162	1970	Egg albumin	Isopiestic equilibrium	Unfolding begins when 5U or 7GuH^+ mol/mol protein are bound. In the D state both species bind equally (33 mol/mol) but mainly by dehydrating the protein. GuH^+ is a more potent dehydrating agent than U.
147	1970	Lysozyme	Ultrafiltration, ORD, viscosity, enzyme assay, -SH determination	N → D transition not accompanied by significant changes in ORD parameters, therefore little disruption of N conformation by ORD criteria. Protein perturbation apparent by $[\eta]$ and S–S stability criteria.

TABLE 10 (continued)

Summary of Recent Experimental Studies of N → D Transitions Induced by Urea and Guanidinium Salts

Reference	Date	System	Experimental technique	Main conclusions
154	1970	Lysozyme	Optical rotation	>35°C an intermediate state exists which has 70% of the degree of the unfolding of the D state. $\Delta C_p > 0$, but smaller than for the complete unfolding.
163	1971	Lysozyme	Calorimetry	Main contribution to ΔH are solvation changes which are only weakly reflected in optical rotation measurements.
86	1971	Lysozyme, ovalbumin	Dilatometry, pH titration	Differences in ΔV of protonation of lysozyme for 6 *M* GuHCl and 8 *M* U, therefore the degree of denaturation differs substantially.
153	1971	Lysozyme	Calorimetry	Calorimetric and van't Hoff values for ΔH are identical for pH induced unfolding in aqueous GuHCl. This implies the absence of intermediate states.
157	1971	B.S.A., ovalbumin, β-lactoglobulin	Ultrafiltration	The protein: U ratios are the same for all proteins (there is uncertainty whether U affects the degree of hydration.) U therefore acts at a common site, i.e., the peptide backbone. Binding is complete within minutes of addition of U, then remains constant during a slow unfolding process.
99	1971	B.S.A.	P.m.r. of water at −25°C	U does not act by large scale dehydration. Upper limit of binding for 50% denaturation is 1U/10 amino acid residues or 65U/mol protein. Unfolding complete at 150 U/mol protein. Folding is a multistep process accompanied by a 10% change in hydration.
165	1971	B.S.A., β-lactoglobulin, lysozyme	Ultrasound absorption	Results cannot be interpreted in terms of conformational changes, but in terms of proton transfer. Ultrasound absorption is not sensitive to conformational equilibria.

TABLE 10 (continued)

Summary of Recent Experimental Studies of N → D Transitions Induced by Urea and Guanidinium Salts

Reference	Date	System	Experimental technique	Main conclusions
145	1971	Lysozyme	pH titration	The reversible titration curve of GuHCl can be predicted on the basis of model pK_a values. The D state is therefore a random coil.
156	1972	Collagen	Optical rotation	U and GuHCl form strong hydrogen bonds with protein.
62	1972	Lysozyme, B.S.A.	Far and near UV absorption, circular dichroism	GuH^+ promotes a conformational rearrangement in the region of the S–S bonds, but helical regions are not affected by GuH^+.
166	1972	B.S.A., ovalbumin, lysozyme	Equilibrium dialysis, O.R.D.	Maximum unfolding at 1 GuHCl/4 amino acid residues. Less unfolding in GuSCN. Gu_2SO_4 produces preferential hydration with a near-native ORD spectrum. Site of interaction of GuH^+ is not clear.
161	1972	Lysozyme	Dilatometry	GuHCl acts through competitive displacement of water which produces unfolding. Further binding takes place to the D state. Complete unfolding takes place at 4.7 *M* GuHCl, but calorimetric data suggest 6 *M* GuHCl for complete unfolding.
103	1973	Alkylvinyl ether – maleic acid copolymer	pH titration, viscosity	Hypercoil → extended (E) state; doubt the concept of nonaccessibility of hydrophobic groups in the hypercoil state – also their complete exposure in the extended state. [η] measurements suggest that U enhances the solvation of the polymer in *both* forms. U acts at apolar side chains and also at backbone sites. Quantitative resolution into additive effects is quite unwarranted at the present time.
87	1973	B.S.A., myoglobin	Dilatometry, pH titration	ΔV mainly reflects the exposure of shielded ionic groups by GuHCl and U: 60 $COO^-/10^5$ g protein are masked in the N form. 8 *M* U and 6 *M* GuHCl exhibit different modes of action.

TABLE 10 (continued)

Summary of Recent Experimental Studies of N → D Transitions Induced by Urea and Guanidinium Salts

Reference	Date	System	Experimental technique	Main conclusions
59	1973	Lysozyme	UV absorption	Complex kinetics at high GuHCl concentrations: two intermediate states, one accumulates during the refolding at low GuHCl concentration, but the reversible N → D transition is a two-state process (this is not the case for cytochrome c). The refolding of reduced lysozyme takes place via faulty folded intermediate states.
160	1973	Lysozyme	Calorimetry	GuHCl binds more strongly to the D than to the N state. Without knowing the degree of protein solvation for at least one GuH^+ concentration, a detailed discussion is pointless. ΔC_p suggests less binding at higher temperatures.
146	1973	Lysozyme	Calorimetry	The S–S cross-linked peptide in GuHCl is a random coil structure, i.e., GuHCl is a good solvent.
61	1973	Poly-L-lysine, Poly-L-glutamic acid, collagen.	Circular dichroism	GuHCl favors locally ordered (E) structures (extended helix), related to polyproline II? U and GuH^+ act stoichiometrically on the carbonyl group. Better bonding at low temperature explains the inverse temperature transitions, e.g., partial cold denaturation of β-lactoglobulin by U. The participation of hydrophobic interactions in the N → D transitions is discounted.
155	1973	Ribonuclease	P.m.r. (histidine protons)	4 his residues involved in the unfolding but they do not unfold at the same GuH^+ concentration, 2 intermediate states are observed. At 3.1 m GuH^+ all 4 his residues are "unfolded."

TABLE 10 (continued)

Summary of Recent Experimental Studies of N → D Transitions Induced by Urea and Guanidinium Salts

Reference	Date	System	Experimental technique	Main conclusions
167	1974	Ribonuclease, α-chymotrypsin, β-lactoglobulin, lysozyme	Optical rotation	U and GuH^+ promote random coil conformation. $\Delta G_{N \rightarrow D}$ varies in linear manner with U and GuH^+ concentration. Both U and GuH^+ act by the same mechanism. $\Delta G_{N \rightarrow D}$ extrapolated to zero concentration of denaturant are given as (kJ mol^{-1}) RNase (pH 6.6): 40.5, lysozyme (pH 2.9: 25.5, α-chymotrypsin (pH 4.3): 34.7, β-lactoglobulin (pH 3.2) 49.
159	1974	Ribonuclease, lysozyme, and 10 others	Densimetry	$\Delta V_{N \rightarrow D}$ small, < 700 cm^3 mol^{-1} for all proteins. All proteins bind GuH^+ preferentially; peptide bonds and aromatic side chains act as binding sites.
M. P. Tombs, personal communication,	1975	α-Lactalbumin	UV difference spectroscopy	Trp residues emerge in two steps, whereas tyr residues emerge in a single step, coincident with the second trp exposure; the N → D transition has a complex pH dependence.

titration curve is sufficiently sensitive to allow the discrimination between different conformations. We would argue that any pH titration results can only indicate that all residues are accessible to the solvent. It has in fact been reported[147] that, judged by ORD (optical rotatory dispersion) criteria, the D state of lysozyme closely resembles the N state. It has also been claimed that the N and D states of ribonuclease are conformationally quite similar.[148] The labile nature of the D state (in ribonuclease) is well demonstrated by the fact that some enzyme activity still exists even in 8 *M* urea without any detectable refolding; on the other hand, the activity decreases sharply before any appreciable degree of unfolding can be observed.[149] In myoglobin the D state has been identified with a random coil only at very high denaturant concentrations[150] and it has been suggested several times that proteins and other polymers maintain some degree of secondary structure in the presence of U or GuH^+.[62,150] At the other extreme, it has been suggested that GuH^+ in fact promotes locally ordered structures in certain homopolypeptides where the N state is an α-helix.[61] These GuH^+ induced structures (which are confined to polymer segments of limited length) have been identified with the polyproline II helix. In this connection it is of interest to note that the polyproline II structure in aqueous solution is in fact *stabilized* by urea.[151] Low concentrations of urea have also been found to promote the recovery of the collagen fold from gelatin (the denatured form of collagen),[152] i.e., U promotes a D → N transition.

As regards the possible existence of intermediate conformations, these have been ruled out on the basis that there is good agreement between the calorimetric and van't Hoff enthalpies of unfolding.[153] On the other hand, such states have been postulated, e.g., in the case of lysozyme above 35°C.[154] At very high GuH^+ concentrations two intermediate states have been observed; one of them is accumulated during the refolding at low GuH^+ concentration.[59] In the case of reduced lysozyme, the D → N process is slow and the observed intermediates are probably incorrectly folded states.

Quite convincing evidence for the existence of intermediates is provided by the p.m.r. spectra of the four histidine residues in ribonuclease as a function of GuH^+ concentration.[155] The signal narrowing to be expected for an N → D process does in fact take place for all four histidine residues, but not at the same GuH^+ concentration. Two intermediates can be observed: one in which his-12 alone is exposed to the solvent and the second one, at a higher GuH^+ concentration, in which only his-105 remains "native", i.e., in a nonsolvent environment. These observations raise doubts as to the validity of the two-state, "all-or-none" model for protein unfolding. On the other hand the sensitivity of n.m.r. is so high that it might just reveal minor deviations from the "two-state" behavior, but such experiments make it possible to chart the pathway of unfolding.*

Turning now to the evidence for U and GuH^+ binding, let us recall the model amide experiments referred to in Section III.C where it was concluded that hydrogen bonding between peptides or between peptides and amides would not be expected to be preferred significantly over hydrogen bonding with water. There are, on the other hand, contradictory claims that U forms "strong" hydrogen bonds with proteins.[156] It has also been stated that the urea: protein ratio for unfolding is the same for all proteins and that therefore urea must act at the common site, namely the peptide backbone.[157] However, here again, there are contradictory reports that, e.g., 8 *M* urea and 6 *M* GuH^+ exhibit different modes of action with different proteins.[86,88] Model peptide studies have been interpreted in terms of a bridging interaction between U and pairs of adjacent peptides,[158] whereas stoichiometric GuH^+ binding at the peptide carbonyl groups has also been suggested.[61] Apart from the peptide group, aromatic side chains have also been implicated as sites for GuH^+ binding.[159] In this connection the thermodynamics of GuH^+ unfolding are of relevance. For the GuH^+ induced unfolding of lysozyme, ribonuclease and myoglobin, $\Delta G^{\sim}_{N\rightarrow D} \sim -50$ kJ mol^{-1}. Model experiments show that a single aromatic group buried in the interior of the protein contributes approximately 12 kJ mol^{-1} to the N state stability, so that only a few residues need to be exposed to the solvent to convert a native globular protein into a "random coil"(?) conformation. Perhaps this can partly reconcile

*Since the completion of this review, Dr. R. H. Pain has drawn our attention to the existence of a stable intermediate conformation induced by GuH^+ in the penicillinase of *Staphylococcus aureus*.[199]

the dramatic denaturation effects with the low quantitative estimates of U and GuH^+ binding.

Several such estimates of the degree of binding have been published. It is generally agreed that the D state has a greater binding capacity than the N state.[85,160,161] The number of U molecules which must be bound per mol protein to induce unfolding has been given as 5 to 7 for egg albumin;[162] for 50% unfolding, 65 mol U/mol serum albumin has been cited.[99] For complete unfolding the corresponding figures for the two proteins are 33 and 150. Enzyme activity studies have led Barnard to suggest that at low ($< 4\ M$) U concentrations, one U molecule per molecule of ribonuclease is bound competitively; at higher denaturant concentrations binding becomes cooperative, resulting in an inactive species.[149] Several workers have pointed out pertinently that it is difficult to obtain *absolute* values for binding processes, since water is the other species which is bound. Thus, with two or more protein states and two small molecule species which are assumed to bind independently to each of these two states, almost any experimental binding isotherm can be fitted.

Once again it is instructive to note the divergent views on the degree of protein dehydration induced by U and GuH^+. Thus Bull and Breese conclude from their isopiestic data that GuH^+ is a potent protein dehydrating agent and urea acts in a similar manner but to a lesser degree; whereas only 33 GuH^+ ions are bound to egg albumin, 700 mol H_2O/mol protein are removed.[162] It is also suggested that the main contribution to the enthalpy of the N → D transition ($\Delta H = -1{,}200$ kJ mol^{-1}) derives from a change in the solvation state of the protein.[163] On the other hand, the low temperature p.m.r. studies of Kuntz and Brassfield suggest that only a 10% change takes place in the degree of hydration.[99] Lee and Timasheff, from a consideration of the A_3 coefficient (see Equation 8), also conclude that binding of U (at the peptide site) is not accompanied by any change in the extent of hydration.[159] Lapanje makes the pertinent observation that without knowledge of the *absolute* value for the degree of protein hydration (A_1 in Equation 8) for at least one GuH^+ concentration, it is pointless to discuss the thermodynamics of unfolding in detail.[160]

The postulate that the interaction of U and GuH^+ with peptide groups alone is responsible for the chaotropic effects of these reagents suggests that the observed salting-in of nonpolar residues, already referred to, is of no relevance to this process. On the other hand, there is plenty of evidence that U and GuH^+ weaken hydrophobic interactions[20] and it is well known that the chaotropic action of U and GuH^+ is not confined to proteins, but can be observed with nucleotides, polysaccharides, lipids, synthetic polymers, and particularly with simpler systems, such as surfactant micelles and dyestuff aggregates where the ordered states are clearly of an entropic origin and closely associated with the hydrophobic hydration of, and interaction between alkyl chains.

Experiments on synthetic copolymers of variously substituted alkylvinyl ethers and maleic anhydride have shown that U acts on the backbone sites, but also on the hydrophobic side chains.[103] A similar alkyl chain length dependence of T_m has been reported for modified polypeptides,[164] and the potency of U in raising the c.m.c. of surfactants is well documented.[36] All these effects can be related to the salting-in power of U for nonpolar residues which, in turn, must in some way be related to the drastic structural modification of the aqueous solvent environment. Let us also remember that the "order" promoting effect of apolar residues in water and the chaotropic effects of U and GuH^+ are quite incompatible[142] and in conformational equilibria the disordering effect of U and GuH^+ usually dominates. Once again the published literature is not too helpful here; some workers discount the participation of hydrophobic effects in N→D processes[61] whereas others state that the urea induced N → D transition (in ribonuclease) is nonspecific, and that urea acts mainly by modifying water–water interactions.[148] Experiments on synthetic model polymers have led Dubin and Strauss to the conclusion that a quantitative resolution into additive effects of urea at backbone and side chain sites is unwarranted at the present time.[103]

We tend to the view that the influence of U on water is well established and that therefore the protein secondary hydration shell B in Figure 6 must be severely perturbed by U; this in turn must affect the primary hydration sphere A. This is surely the reason for the ability of U to "salt-in" apolar molecules, i.e., U acts by reducing the entropic barrier associated with hydrophobic hydration. On the other hand, it also seems sensible that a highly polar molecule, such as U, would interact with other dipoles or charged

residues, but whether it does so in a manner radically different from that of the other competing polar molecule–water is not clear.

In summary then and neglecting secondary binding effects, we remind the reader once again that a polypeptide becomes a protein only by virtue of its association with water. On the other hand, U has the ability of turning water into a completely different solvent by reducing the three-dimensional order which, we believe, is a necessary feature for the maintenance of the N state. Thus in order to understand fully the role of U and GuH^+ in biopolymer unfolding, we must devote our efforts more to an understanding of the B hydration sphere rather than a study of direct binding of these species to the macromolecules.

E. Conformational Effects Induced by "Typically Hydrophobic Solutes"

In the preceding sections we have suggested certain fundamental differences in the conformational changes promoted by ions on the one hand and U and GuH^+ on the other. There exists a third group of protein perturbants – water soluble, monofunctional alkyl derivatives, such as alcohols, ketones, ethers, amines, and tetraalkyl substituted ammonium salts. Evidence has accumulated over recent years that in aqueous solution these substances behave as "typically hydrophobic solutes", i.e., their solution thermodynamics at limiting concentration closely resemble those of alkanes. Most of the protein conformational studies involving this class of perturbant have been performed in water–alcohol mixtures (including some substituted alcohols, e.g., 2-chloroethanol and ethoxyethanol) and we shall therefore confine ourselves to a discussion of these mixtures. We predict, however, that whatever conformational effects are induced by alcohols would also be observed in aqueous solutions of the other monofunctional alkyl derivatives listed above. Indeed some rather interesting effects on protein charge and solubility are produced by tetraalkylammonium halides which appear to exhibit the dual functions of ions and hydrophobic molecules.[16] The possible functional similarity between these ions and biologically active species, such as acetylcholine and various drug molecules has been mentioned[168] but so far no detailed conformational studies involving such species have been reported.

At the outset it must be stated that the most informative way of studying the effects of hydrophobic perturbants on proteins is by the use of hydrocarbons. Several such studies are on record[169-178] but, although of general interest, most of the available data were either obtained with very complex systems and/or are of too low a precision to enable them to be used for quantitative thermodynamic evaluation. The reports by Wishnia and his colleagues are exceptions and provide a valuable insight into the thermodynamics of folded protein states.[172-178]

In general it was found that apolar ligand binding to globular proteins could be described by two equilibria relating to different, noninteracting binding sites. One of these (in the cases of β-lactoglobulin, ferrihemoglobin, and ferrimyoglobin) is a high affinity site in the interior of the protein and is usually very sensitive to ligand stereochemistry. Thus a clear distinction exists between the binding thermodynamics of neopentane and *n*-pentane to β-lactoglobulin. The suggestion has been made that the solubilization of alkane may reduce some internal stresses in the protein interior which are caused during folding and result in suboptimal hydrophobic interactions. The other site(s) appear to be of lower affinity, less specific, and capable of promoting multiassociation.

By extending their studies to include halogenated hydrocarbons of different sizes and shapes, Wishnia and his colleagues[177] have been able to show that the volume of the high affinity internal binding site in β-lactoglobulin is about 200 cm^3 mol^{-1} which is able to accommodate 2 mol of toluene or α,α,α-trifluorotoluene, but only 1 mol of hexafluorobenzene. In addition to characterizing the binding equilibrium in thermodynamic terms, the protein–ligand interactions have also been studied by ^{19}F n.m.r.[176] and the existence of two distinct sites has been confirmed. In addition to considerable differences in affinity and specificity, marked differences in the kinetics of binding have been established.

It is our feeling that, as our understanding of folded states and refolding mechanisms improves, the significance of Wishnia's work will find greater appreciation. On the other hand, these publications highlight the limited scope of alkane studies; the only available experimental tool is that of solubility, where the errors must always be such as to introduce considerable uncertainty into the

thermodynamic quantities derived by van't Hoff procedures. Because of the nature of the molecules and low concentrations involved, spectroscopic techniques for locating "bound" species are precluded, as are also chiroptical and relaxation methods.

To overcome these experimental limitations some workers have resorted to the use of surfactants which, so it is claimed, act on proteins in a similar manner as hydrocarbons, i.e., by the solubilization of apolar residues. While recognizing that protein/surfactant interactions provide an interesting and useful area of study in their own right, it is our view that an extrapolation from surfactant to alkane is completely unwarranted and is unlikely to improve our understanding of the subtle role played by nonpolar amino acid residues in conformational processes.

As mentioned above, the same strictures need not be applied to the use of low molecular weight alcohols in studies of hydrophobic effects. As in the previous section, we begin this discussion with a summary of the relevant properties of alcohol–water mixtures since these constitute the common feature in the ternary protein systems. The reader is referred to Section III.B. which reviews the evidence for hydrophobically induced ordering processes in water, referred to as hydrophobic hydration. The spatial and orientational arrangements of water molecules induced by alkyl groups are likely to be complex[28] (the clathrate model may only be a very rough approximation of these effects). Since in dilute solution the hydrophobic hydration shell must exist in equilibrium with "unperturbed" water – unless hydrophobic hydration is a very long range effect – we can conceive of a region, fairly close to the hydrophobic hydration shell, in which the distribution of molecular orientations (and perhaps intermolecular distances) gradually changes, finally to reach the distribution characteristic of liquid water. The distance over which this change in the molecular orientations takes place is probably on the order of several molecular radii. Hydrophobic hydration is thus seen to be a long-range effect at least in terms of protein hydration and is likely to extend into region B of Figure 6.

The pair interaction between two alkyl groups in an aqueous medium is rather complex and not altogether compatible with predictions based on the simple model of the hydrophobic bond.* The experimental evidence suggests that some water molecules, affected by two or more alkyl groups, undergo a further ordering process, but it is also probable that the interaction between hydrated alkyl groups takes place in such a way that fewer water molecules are required for the formation of cooperative hydration spheres than in the hydration spheres of the isolated alkyl residues. There appears to be a critical concentration c* of alkyl residues, or rather of $-CH_3$, $>CH_2$ and $-CH$ groups, beyond which hydrophobic hydration becomes progressively cooperative. At this concentration the mixture becomes subject to considerable concentration fluctuations which are typically associated with the phenomenon of lower critical demixing, i.e., by "weakening" the water–water interactions, a critical temperature is reached at which the water can no longer maintain the separation of alkyl groups and hence phase separation occurs. At even higher concentrations of alkyl groups the solution loses its unique aqueous character and behaves more like "normal" mixtures of small polar molecules, capable of interacting by hydrogen bonding. It is in this concentration range that hydrophobic aggregation phenomena, such as micelle formation of surfactants, take place. There are factors such as the alkyl group configuration, internal degrees of freedom, the nature of the polar group, which influence c*, and its temperature dependence (these factors also influence the critical micelle concentration of surfactants), but such influences are of a minor nature.

Bearing in mind then the apparently subtle concentration effects which nevertheless can change not only the magnitude, but also the *nature* of the solvent component interactions, it is likely that in the system water–protein–alcohol, the existence of the critical solvent composition c* will be reflected in the conformational behavior of the protein. It can also be predicted, and is borne out by experiment, that the alcohol effects on protein conformational stability are much more complex in terms of solvent composition and temperature dependence than the "simple" chaotropism induced by urea and GuH^+. These two predictions are of course based on the premise that the main sites of interaction between the mixed solvent, or the separate solvent components, and

*For a state-of-the-art account of the hydrophobic interaction the reader is referred to Reference 20.

the protein are apolar side chains (val, leu, ile, phe) and possibly, but to a lesser degree, apolar portions of side chains which carry terminal polar groups (such as lys, glu, ser, thr, trp, tyr).

The following pages review what we consider to be significant investigations into the conformational effects produced on proteins by alcohol–water mixtures. At first glance much of the available experimental information suggests that alcohols lower T_m of the N state and give rise to a typical, nonspecific unfolded D state. The reasoning is based once again on results from ΔG_t° studies with model compounds. Figure 13 indicates that ethanol (and dioxan) "salt-in" leucyl side chains. Thus the alkyl group on the alcohol is said to interact with the apolar side chains in the interior of the protein and thus to facilitate unfolding.[34,159,179-183] As before, in the discussion of ionic and urea effects, the problem can be tackled via the specific binding or the more general salting-in and out approaches. In addition we have the knowledge that, in this particular case, the solvent mixture itself is of the utmost complexity, and it must be considered whether such inter-solvent effects can be adequately included in the two approaches used so far. We shall return to these subtle solvent effects later.

Several theoretical and model studies have examined the effects of organic cosolvents on protein stability. For instance, the Langmuir model, treating solvent binding as adsorption on an elastic linear polymer chain, has been employed to calculate the effect of mixed solvents on the conformational stability of proteins.[184] At its simplest, the model polymer is composed of polar and nonpolar segments for which allowance is made in the partition function. A polar segment has one equivalent and independent adsorption site for binding a cluster of polar (water) molecules, while each nonpolar segment has one site for a cluster of nonpolar solvent (alcohol) molecules. Adsorption can occur only on sites in the D conformation, since sites in the N state are inaccessible to solvent. It is also assumed that inaccessible adsorption sites in the N state become fully accessible in the D state. The total protein surface area can then be calculated and from this and the distribution of polar and nonpolar sites on the protein exterior, various assumptions about magnitudes of solvation interactions and their influence on the stability of the N state allow the calculation of an unfolding isotherm. The various parameters and quantities introduced into this theoretical treatment can be related to experimentally derivable quantities such as $(\partial w_3/\partial w_2)_{T,\mu_1,\mu_3}$ (see Equation 8), but at best a crude form of rationalization of the mixed solvent effect on unfolding can be achieved. Thus, whereas the observed behavior of β-lactoglobulin in water-2-chloroethanol mixtures can be fitted by such a statistical adsorption model, the absence of unfolding with insulin cannot, although both proteins show very similar solvent composition dependence of $(\partial w_3/\partial w_2)_{T,\mu_1,\mu_3}$.

One weakness of the above and other similar approaches is that water-cosolvent interactions are completely ignored. The postulates that the protein is a Langmuir solid with independent adsorption sites and that the two adsorbate species form a statistical mixture must also be of doubtful validity.

It might have become apparent to the reader that in our view (and we are encouraged by similar assessments from researchers with more extensive experience in the conformational behavior of proteins) sophisticated statistical thermodynamic treatments, based on discrete sites, specific interactions, specific conformational states, etc. are premature in the light of our somewhat sketchy knowledge, and can at best be used to fit experimental results, and provide comparisons between the behavior patterns of different protein and solvent systems.

We shall therefore return to the discussion of the available experimental information in terms of N and D and possibly other conformational states.

On the basis of Figure 13 it can be predicted that increasing concentrations of ethanol should promote secondary structure, since peptide groups are preferentially salted-out. At the same time apolar groups are salted-in, so that tertiary structures, based on internal hydrophobic contacts, should be destabilized.

The fact that alcohols denature globular as well as fibrous proteins is well established. In agreement with the suggestion that the alcohol acts by dissolving out apolar groups from the interior, it has been observed that, both c* and the concentration of alcohol required for unfolding are inversely proportional to the number of CH_2 groups in the alkyl chain.[180,182,185-187]. It has also been suggested that for $T_m > 60°C$, the combined effects of two alkanols on T_m of ribonuclease are

additive,[188] but curiously, no such additive behavior can be observed at lower temperatures.

The considerable body of experimental data on alcohol denaturation of proteins prompts the question why it is that hydrocarbons which have also been shown to interact strongly with apolar residues in the interior of proteins[175-178] do not appear to destabilize the N state.

Also of interest, although probably not of immediate relevance to the present discussion, are the observations of Gerlsma that polyhydric alcohols in aqueous solution are potent stabilizing agents for N states of globular proteins.[179, 188-190] To our knowledge, no binding studies have been performed with aqueous mixtures of polyols and simple carbohydrates, but the stable orientation specific hydration states of these molecules[191] might well be implicated in such stabilizing effects.

We have so far concentrated on the hydrophobic contribution to the N⇌D equilibrium, and only mentioned briefly that since alcohols salt-out peptide groups, one might expect alcohols to promote the stability of secondary structures which are characterized by interpeptide hydrogen bonds. It is well established that at high alcohol concentrations proteins exist in the D state, but at the same time contain considerable regions of helical structure. Circular dichroism measurements have shown[192] that these ordered regions form by noncooperative mechanisms with increasing alcohol concentration and the question has been posed whether these ordered structures are necessarily α-helical. One fact is certain, however, the D states induced by urea and ethanol may resemble one another in being coil-like hydrodynamically, but in the presence of ethanol there are regions of peptide chains with a high degree of structural order, a feature which is not usually found in the presence of high concentrations of urea.

That such structures can be identified with the α-helix is suggested by the effects of alcohols on T_m of polymers which possess no tertiary structure to partially obscure the experimental results, e.g., poly-L-ornithine and poly-L-glutamic acid.[192] With globular proteins we apparently have to deal with two competing processes: the weakening of tertiary structure with the subsequent (or simultaneous?) stabilization of some form of secondary, helical structure. This is well demonstrated by the thermal unfolding behavior of lysozyme shown in Figure 14. The lower curve shows the effect of increasing concentrations of *n*-propanol on T_m as monitored by the difference spectrum at 294 nm. The family of curves is characteristic of the cooperative N→D transition with increasing concentrations of perturbant producing an increase in ΔT_m. The upper curve shows the corresponding reduced mean residue rotations (m′) at 233 nm, as obtained from ORD measurements; also included are some values for the Moffit–Yang b_o parameter. Comparison of the two sets of results indicates that different processes appear to be taking place simultaneously. Thus at low concentrations of propanol, the $\epsilon(T)$ and $m'(T)$ curves are qualitatively similar and their midpoints coincide. As the propanol concentration increases, well-developed maxima appear in $m'(T)$ which do not correspond to the midpoint of the N→D transition. In addition, b_o values do not correspond to those usually associated with the "random coil" (0 to +50) but tend towards the α-helix values.

The concept of the simultaneous melting of tertiary structure and increase in secondary structure is, at first sight, quite acceptable in terms of solvent component interactions with individual protein residues and has been observed and discussed by several workers.[125] These dual (or multiple) solvent effects raise certain problems:

1. An exact thermodynamic analysis of the overall unfolding process in terms of energetic and entropic contributions must be hazardous.
2. Attempts to perform such an analysis have led to the discovery of a third, and much more subtle, solvent effect.

Thus from the alcohol induced spectral changes discussed above, the van't Hoff enthalpy of denaturation of lysozyme can be derived and is shown in Figure 15. According to the authors,[186] the "peculiar variation of ΔH with alcohol content is unexpected" and they suggest that the origin of this effect is of an entropic nature. However, their analysis of the situation leads them to the "seemingly conflicting conclusion that upon increasing alcohol concentration, the entropy of the globular → random coil transformation increases while, simultaneously, the entropy of the helix → random coil transformation decreases." They state however, that the "binding of alcohol to the non-polar side chains which become

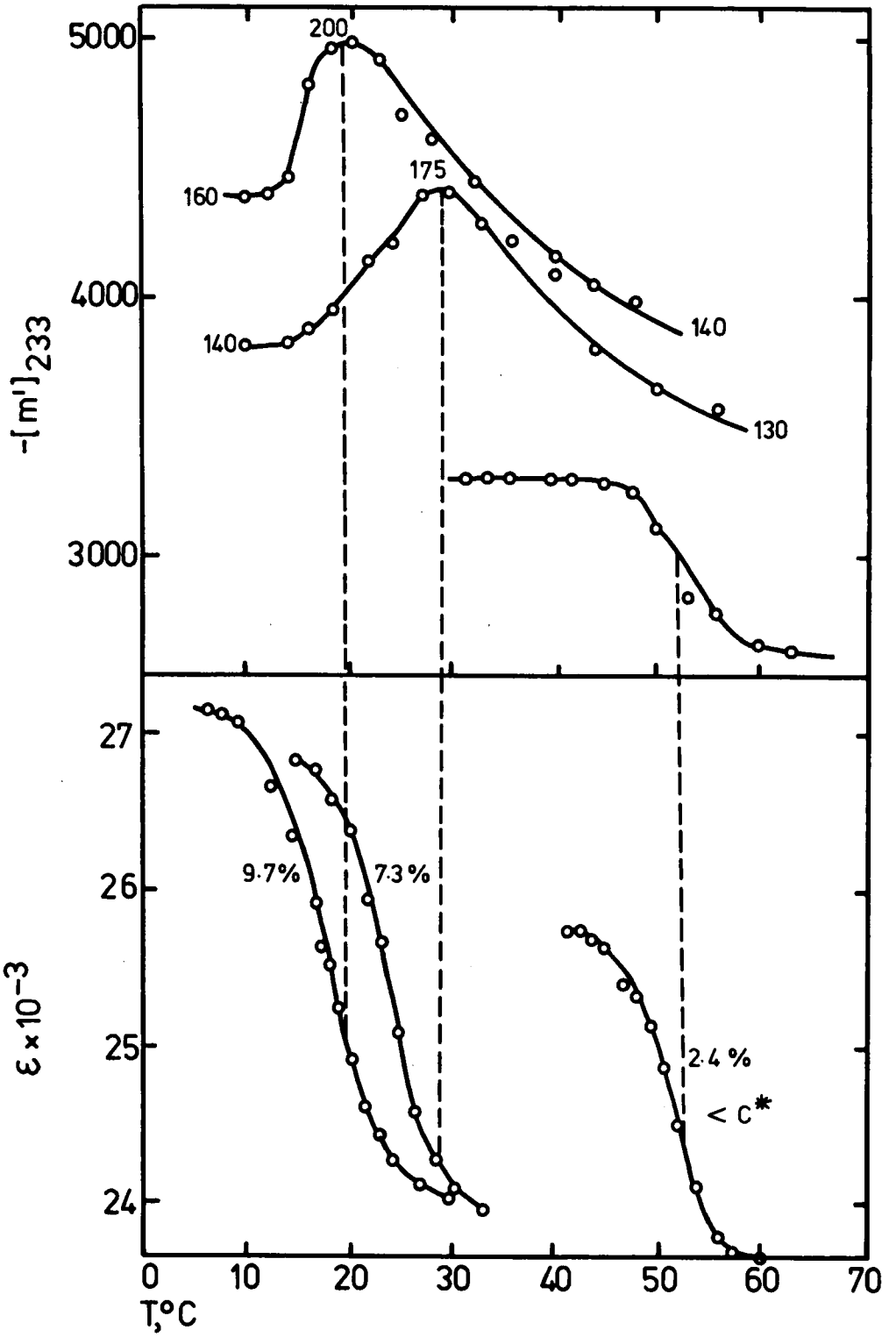


FIGURE 14. *n*-Propanol induced changes in the thermal unfolding of lysozyme.[186] Bottom: Optical density (ϵ) at 294 nm monitors N→D transition. Top: Reduced mean residue optical rotation [-m] at 233 nm measures the degree of secondary structure. Values of the Moffit-Yang $-b_o$ parameter are indicated in several places of the [-m'] (T) curves. Only at the lowest PrOH concentration (<c*) do the two curves coincide.

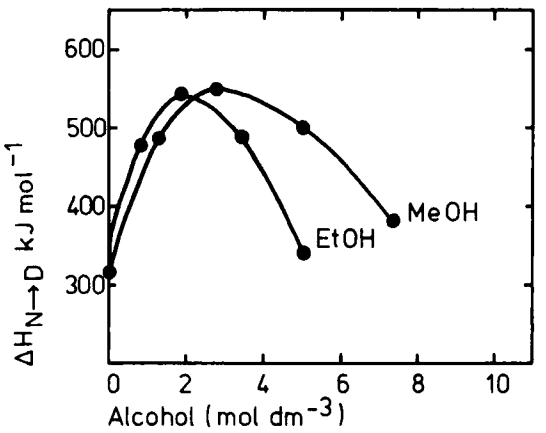


FIGURE 15. The van't Hoff $\Delta H_{N\rightarrow D}$ for the alcohol induced unfolding of lysozyme[186] as a function of alcohol concentration.

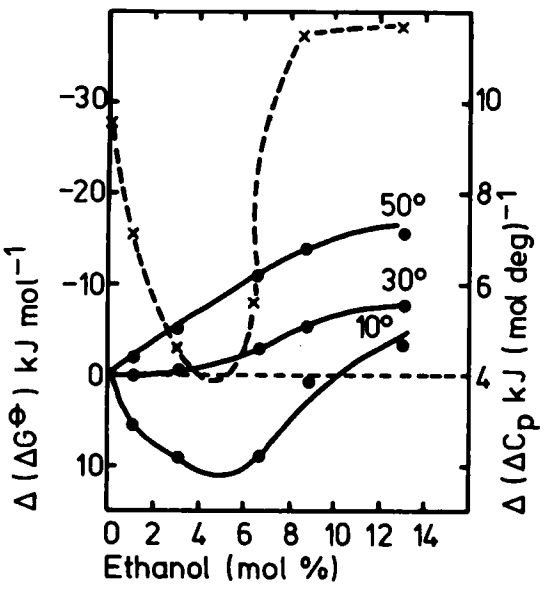


FIGURE 16. The effect of ethanol on $\Delta G^{\ominus}_{N\rightarrow D}$ of ribonuclease at 10, 30, and 50°C (•), and on ΔC_p at 40°C (x). (For explanation, please see text.) (Adapted from Brandts, J. F. and Hunt, L., The thermodynamics of protein denaturation. III. The denaturation of ribonuclease in water and in aqueous urea and aqueous ethanol mixtures, *J. Am. Chem. Soc.*, 89, 4826, 1967.)

exposed during the former transition, coupled with *water organization effects* satisfactorily explains the above findings." Although this statement seems to us somewhat optimistic, nevertheless the problems have been posed and brought out into the open for discussion. A careful examination of the literature reveals that the effects of alcohol–water mixtures are indeed very subtle and that, e.g., low concentrations of ethanol, especially when coupled with low temperatures, produce effects which are hard to reconcile with the models so far discussed. Thus it has been reported that at 0°C, but not at 25°C, the native form of glutamate decarboxylase is preferentially stabilized by low concentrations of ethanol.[193] Similar stabilizing effects have been noted in the denaturation kinetics of trypsin.[194]

However, it was Brandts and Hunt who, on the basis of studies of the ribonuclease unfolding by aqueous ethanol (and by aqueous urea), first pointed out explicitly that the simple concept of the dissolving out of apolar residues could not be reconciled with the experimental data.[148] First of all, Brandts and Hunt highlighted close coupling of cooperative *solvent* order–disorder processes with the maintenance of N states, i.e., that there exists a temperature of maximum protein stability, T_{max}, and that the N→D transition can be induced by *low* as well as high temperatures. They then showed that the effect of ethanol on the temperature dependence of $\Delta G_{N\rightarrow D}$ was very different from the effect of pH. The mixed solvent effect on $\Delta G_{N\rightarrow D}$ was defined as follows:

$$\Delta(\Delta G^{\ominus}) = \Delta G^{\ominus}(x_3) = \Delta G^{\ominus}_0,$$

where x_3 is the mol fraction concentration of ethanol and $\Delta G^{\ominus}_0$ is the corresponding standard free energy of unfolding in the absence of alcohol but under identical conditions of temperature and pH (see footnote on p.4830 of Reference 148). Figure 16 provides one of the most convincing demonstrations of the complexity of the solvent involvement in N→D transformations. It shows quite clearly that at 10°C a dilute aqueous ethanol solution has a pronounced stabilizing effect on the native protein;* at 50°C the same solvent mixture induces destabilization (compared to water) to about the same extent; i.e., $\Delta(\Delta G^{\ominus})$ is approximately equal in magnitude but of opposite sign at 10°C and 50°C. It is readily seen from Figure 16 that $\Delta H^{\ominus}$ must assume a very complex x_3 dependence and this is indeed the case (see Figure 15). However, the most striking feature of the transi-

*Alternatively the solvent can be regarded as promoting another protein conformation, the E state, which bears a close resemblance to the N state.

tion is ΔC_p, also included (at 40°C) in Figure 16. It is seen that ΔC_p in 5 mol % ethanol falls to less than half of its value in pure water and with increasing ethanol rises steeply to regain its former value. If it is remembered that current doctrine associates a large positive ΔC_p with the exposure to the solvent of previously hidden apolar residues, then Figure 16 suggests not only that in 5 mol % ethanol the tendency of apolar residues to become unfolded is very much reduced, but also that the solvation sphere surrounding exposed apolar residues changes drastically as x_3 is increased. It must also be remembered that many globular and all fibrous proteins in their N states exhibit appreciable apolar regions on the surface, and that these regions, exposed to the changing solvent composition, are likely to act as nucleation sites for solvent stabilization or destabilization effects, whichever the case may be.

The results of Brandts and Hunt, although not unique, are the most explicit examples of the complex solvent effects produced by mixtures of water with low concentrations of "hydrophobic" molecules. Such effects had previously been observed and discussed by solution physical chemists,[128,195,196] but the information had apparently not reached the biochemist studying order–disorder processes in biopolymers. As an example of the close analogy between the response of a globular protein and a very simple molecule argon, to water–alcohol mixtures of varying composition, the reader is referred to Figure 17 which demonstrates changes in the free energy of solution of argon with increasing ethanol concentration. Thus $\Delta(\Delta G^{\ominus}) > 0$ signifies "salting-out" of argon by ethanol. These findings have recently been confirmed and also established for the water-*tert*-butanol solvent system where the "salting-out" effect is even more pronounced.[195]

One cannot help but be struck by the similarity of two such dissimilar molecules as argon and ribonuclease in responding to the properties of the solvent mixture, even to the range of solvent composition (allowing for the different temperatures) at which the protein stabilization turns into destabilization and salting-out of argon into salting-in, respectively.

Apart from the very comprehensive studies summarized above, Brandts and Hunt also investigated the urea induced unfolding of ribonuclease.[148] Detailed comparisons of the thermodynamics and the ORD changes which characterize

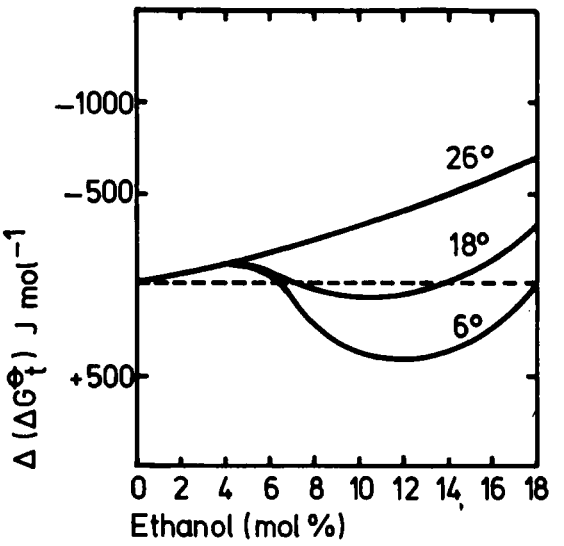


FIGURE 17. ΔG_t^{ϕ} (water→aq. ethanol) of argon at 6, 18, and 26°C. Whereas the "normal" effect of ethanol is to salt-in argon, at low ethanol concentrations and low temperatures, argon is salted-out. (Adapted from Ben-Naim, A. and Baer, S., Solubility and thermodynamics of solution of argon in water + ethanol system, *Trans. Faraday Soc.*, 60, 1736, 1964.)

these processes show beyond any doubt the fundamental difference in the mode of action between these two solvent systems with the aqueous urea giving a simpler picture. The possibility must certainly be explored that the D states produced are not identical, and are certainly not "random coils", although the N→D transitions are fully reversible in all cases.

We have dwelt at some length on the ribonuclease studies by Brandts and Hunt because they constitute a most important step forward in our appreciation of complex solvation effects in biopolymer conformational stability. However, even eight years later, the implications have not yet been fully appreciated by protein chemists (e.g., Reference 186) and it is still common to read that hydrophobic solutes denature proteins by dissolving out apolar residues.

The conclusions of Brandts and Hunt can now be somewhat extended and we may look for the reasons why the solvent medium effects on globular proteins should simulate so closely those on argon (and other simple molecules). The thesis has already been advanced earlier that in dilute solutions of alcohols (especially at low temperatures), the water–water interactions are effected in a way which is commonly described as "water structure

promotion" and that this must be achieved by minor changes in the orientations of, and distances between water molecules. If, therefore, at any given temperature water maintains the N state, then an aqueous solution of alcohol in the concentration region ($\leqslant c^*$) where this type of structure promotion is observable should provide an additional contribution to the stability of the N state even to the extent of promoting an even "better" folded, E state.

Apart from the examples already cited, this extra stabilization of a native, or E state, can be observed (if looked for!) in a number of other protein systems. Thus, Figure 18 shows the effect of *tert.*-butanol on some spectral properties of myoglobin and α-chymotrypsinogen at 25°C. In both cases an increase in the relevant parameters is observed at low alcohol concentrations. The chymotrypsinogen unfolding was monitored at 292 nm, i.e., changes experienced by tryptophan chromophores. Since the exposure of chromophores during the N→D transition leads to a decrease in $\Delta\epsilon$, the initial increase at low *tert.*-butanol concentrations may correspond to a tighter folding with initially exposed tryptophan residues becoming partly shielded from the solvent.*

It is interesting that Herskovits et al.[180] briefly refer to, and then reject the findings of Brandts and Hunt, mainly "in view of the..... assumptions involved in the derivation of the equations used." They then state that "it is gratifying that a single ΔH value, based on the literature, in most cases predicts a satisfactory set of denaturation parameters..... for a family of related denaturants such as the alcohols." In the light of the evidence now available, this is to us an untenable conclusion.

As a final example of the role of subtle solvent effects on conformational equilibria, we examine the lysozyme unfolding studies of Timasheff and his colleagues.[125] The perturbants were chosen for their "helix-inducing" properties and include ethylene glycol, 2-methoxyethanol and 2-chloroethanol. By means of CD in the far ultraviolet it was shown quite convincingly that these solvents at relatively high concentrations indeed promote α-helical conformations. "Preferential" solvent binding was calculated from light scattering and

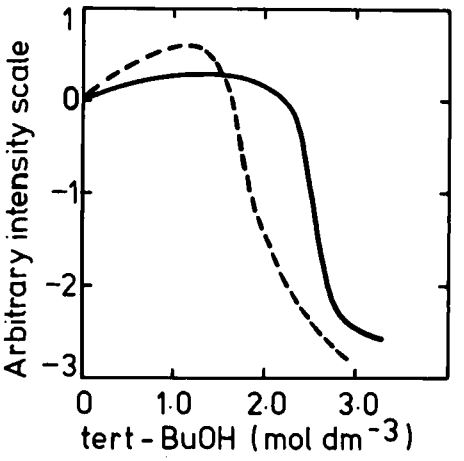


FIGURE 18. The *tert.*-butanol induced isothermal unfolding of myoglobin (—) and α-chymotrypsinogen (- - -), as monitored by UV difference spectroscopy. Whereas the general effect of *tert.*-BuOH (and other alcohols) is one of destabilizing the N state, low concentrations ($< c^*$) promote a more ordered or more tightly folded conformation. (From Herskovits, T.T., Gadegbeku, B., and Jaillet, H., On the structural stability and solvent denaturation of proteins. 1. Denaturation by the alcohols and glycols, *J. Biol., Chem.*, 245, 2588, 1970. With permission.)

differential refractometry data and the preferential binding of 2-chloroethanol (or water) by lysozyme is shown in Figure 19. The complexity of the curve is explained by the N→D transition, i.e., there are two macromolecular species, N and D, both of which possess certain numbers of binding sites for water and 2-chloroethanol. In terms of numbers of molecules of both solvent species bound to a molecule of protein, this provides four adjustable parameters for fitting the unfolding equilibrium. Since $(\partial m_3/\partial m_2)_{T,P,\mu_3}$ is related to protein hydration by

$$\left(\frac{\partial m_1}{\partial m_2}\right)_{T,P,\mu_1} = -\frac{m_1}{m_3}\left(\frac{\partial m_3}{\partial m_2}\right)_{T,P,\mu_3},$$

Timasheff et al.[125] tried to estimate a "reasonable" value for the term on the left hand side and hence to arrive at actual numbers of molecules of bound species per mol protein.[125] However, at this stage we remind the reader of the uncertainties inherent in such estimates (see Table 9), so that he may judge for himself the value of such a procedure.

*Increases in $\Delta\epsilon$ are also produced by a change in the polarizability of the solvent medium and can be calculated and allowed for. The effects in Figure 18 are much more marked than would be expected simply on this basis.

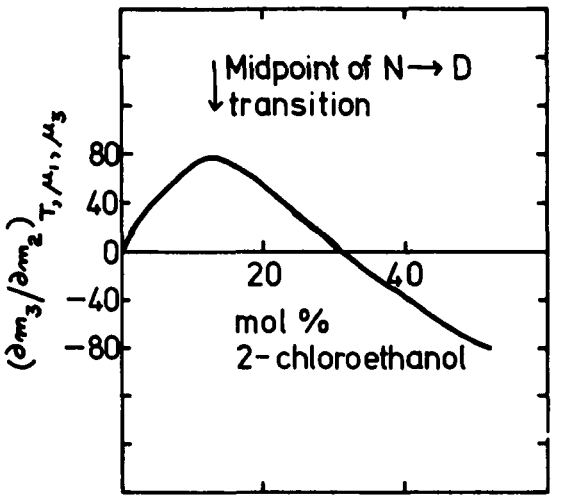


FIGURE 19. The differential binding of water and 2-choloroethanol by lysozyme, determined by differential refractometry and Equation 8. (Adapted from Timasheff, S.N., Protein-solvent interactions and protein conformation, *Acc. Chem. Res.*, 3, 62, 1970.)

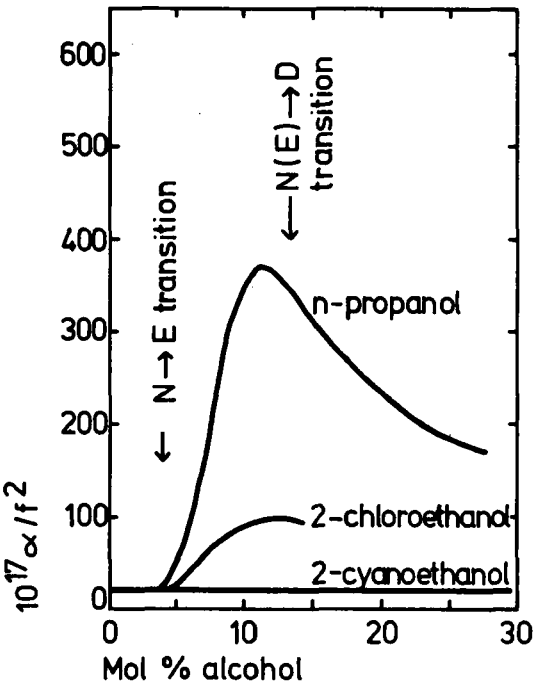


FIGURE 20. Ultrasonic absorption (α/f^2) of alcohol-water mixtures at 25°C and a frequency f = 70 MHz; α is the absorption coefficient. The characteristic behavior common to these mixtures is not shown by 2-cyanoethanol which is not a "typically hydrophobic", but a bifunctional molecule. The alcohol concentrations corresponding to the two observed lysozyme transitions are indicated. (Adapted from Blandamer, M. J., Hiddon, N. J., Symons, N. J., and Treloar, N. J., Ultrasonic absorption properties. Part 1. Mixtures of water and substitute alcohols, *Trans. Farad. Soc.*, 65, 1805, 1969.)

To confirm the presence of the unfolding of lysozyme by 2-chloroethanol, Timasheff extended his CD studies to the region above 250 nm. The general effect produced by 2-chloroethanol is to reduce the signal intensity (which is due to aromatic residues and disulfide linkages), but at low concentrations (~5 mol %) an enhancement in the intensity of the 280- to 290-nm band was observed, compared to the value for water. This again seems to indicate a loss in mobility of one or several chromophores which in the N state are normally exposed to the solvent. We now have two significant concentrations of 2-chloroethanol: 5 mol % at which the enhancement in the CD intensity is observed (N→E transition ?) and 12 mol % where $(\partial m_3/\partial m_2)_{T,P,\mu_3}$ shows a maximum (see Figure 19) and where viscosity data place the midpoint of the N→D transition. By comparison, Figure 20 shows the concentration dependence of the ultrasound absorption by water-2-chloroethanol mixtures. This type of curve is characteristic of water–alcohol mixtures in general (*n*-propanol is included in Figure 20) and is related to the existence of microheterogeneous regions induced by concentration fluctuations, i.e., the system behaves as a two-phase system on a microscopic scale.[197] The water structure stabilization effects, already referred to, give rise to the initial horizontal part of the curve. It appears that microinstability sets in at about 5 mol % and reaches a maximum at 12 mol %, beyond which the mixture loses its "typically aqueous" character. Unfortunately thermodynamic data are lacking for water-2-chloroethanol mixtures, but what few indications there are available of complex solvent behavior should be carefully considered before it is assumed that, for the purposes of binding by proteins, the solvent is a statistical mixture of two molecular species.

The type of behavior shown in Figure 19 is now seen to be common to simple and complex ternary systems (see for instance Figures 16 and 17). A recent study of the thermodynamics of transfer of urea from water to aqueous tetrahydrofuran (THF) shows that $\Delta G_t^{\ominus}$, which is initially negative, undergoes a minimum at about 8 mol % THF and changes sign at 12 mol %.[198] The authors rightly emphasize that it is therefore impossible to describe $\Delta G_t^{\ominus}$ for urea in terms of a simple preferential solvation model. If such a cautionary

remark is in order for simple ternary systems, then a meaningful interpretation of $(\partial m_3/\partial m_2)$ in protein-containing ternary systems presents even greater difficulties, particularly in mixed solvents where the solvent components themselves exhibit marked anomalies from normal solution behavior.

VII. SUMMARY AND CONCLUSIONS

In this review we have tried to demonstrate that protein conformational stability is closely linked with the properties of the solvent system. The direct action of solvent components on protein residues can be classified in terms of a number of reasonably well-defined types of interactions: electrostatic, dipolar, hydrogen bonding, and hydrophobic. However, protein conformations are stabilized or destabilized not just by nearest neighbor interactions, but rather more subtle solvent effects play a crucial role.

One of the basic properties of proteinaceous systems is the "degree of hydration", but there is as yet no unequivocal way of defining this. Certainly different experimental techniques "see" different degrees of hydration. This uncertainty in the degree of hydration is reflected in the determination of binding of other solvent components since the available experimental techniques only provide a measure of binding *relative* to that of water. Furthermore, it is not yet clear what binding sites exist for many of the small molecule species. In particular, it is surprising that binding effects are so often found to be additive. The implications of the lyotropic (Hofmeister) series in binding specificity also awaits further insight.

The relationship between binding and conformational perturbations is not yet clear and in particular the effect of hydrophobic species on the stability of N states and the promotion of specific D states presents unsolved problems.

It is now clear that complex intersolvent effects, where they exist, may provide valuable clues to the solvent involvement in the promotion of certain conformational states, and valuable information about complex solvent effects has been and will continue to be derived from comparisons between proteinaceous and model systems.

Finally, a survey of past experimental endeavors, many of them of great intrinsic value, shows that the diversity of proteins, experimental conditions such as pH, temperature, type of solvent, experimental techniques used, and the general lack of coordination between different investigators working in closely allied fields, etc., has produced a veritable jungle of data and hypotheses; a more purposeful, joint attack on some of the problems discussed here may well be timely and yield valuable dividends.

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