Hydration of Macromolecules

Abstract. *Frozen protein and nucleic acid solutions at −35°C show relatively narrow (100 milligauss) proton nuclear magnetic resonance signals which are assignable to water that is sufficiently mobile to reduce the dipolar broadening normally associated with solids. Hydration was found to be 0.3 to 0.5 gram of water per gram of protein. Nucleic acids are three to five times as hydrated as proteins. Conformational changes in solution produce detectable changes in linewidth or amount of "bound" water, or both. The very fact that the water signals can be observed by high resolution nuclear magnetic resonance suggests that it is not "ice-like" in any literal sense, although it is clearly less mobile than liquid water at the same temperature. A simple model is described which considers both surface hydration and trapped water.*

We describe a method for estimating the amount of water bound to macromolecules in solution. We have observed that frozen aqueous solutions of proteins, nucleic acids, and other large molecules show broad, low-amplitude proton magnetic resonances at temperatures well below the freezing point of water (Fig. 1). These signals are clearly due to water protons because they are greatly reduced (>90 percent) when D_2O is the solvent; they are centered at, or near, the normal water chemical shift; they are too large to be ascribed to the exchangeable protons of the solutes. Ice itself has a resonance line much wider than those described here. Water and salt solutions show no signals below their freezing (eutectic) points when our techniques are used.

Several lines of evidence suggest that the water is, in some sense, "bound" to the macromolecule. (i) The amount of water detected varies linearly with the concentration of the solute. (ii) The degree of hydration agrees reasonably well with amounts of bound water determined by other methods. (iii) The linewidths of these signals are much greater than those observed for liquid (supercooled) water at the same temperature. (iv) The linewidth and degree of hydration reflect the molecular conformation present in the unfrozen solution.

Our experiments were performed on conventional Varian A60-A and HA-100 spectrometers, equipped with standard variable temperature units. Signals were improved by use of the Varian C-1024 signal averager. The spin decoupler was used in its high-field mode. The averager also provided a 2500-hz sweep width for the A60-A spectrometer. Temperatures were measured with methanol samples and were constant to ± 2°C. Sample spinning was not needed.

The protein solutions (5 to 10 percent) were made up volumetrically with salt-free crystalline preparations of the following: bovine serum albumin, grade A, and hemoglobin, grade A (Pentex); ovalbumin, grade V, and lysozyme, grade I (Sigma); and alpha chymotrypsin (Nutritional Biochemicals). Gelatin was obtained from Baker & Adamson. The only added electrolyte was KCl (0.001*M*), unless otherwise noted. Actual protein concentrations were determined spectrophotometrically after completion of the NMR experiment. Transfer ribonucleic acid (tRNA) samples were provided by J. Fresco. The solutions were normally kept at room temperature until use. Then they were quickly frozen in liquid nitrogen, permitted to warm slightly, and placed in the spectrometer to equilibrate for some minutes. Results were independent of freezing method provided that it was not done too slowly. Aside from some supercooling, the same signals were found by approaching the same temperature from either side.

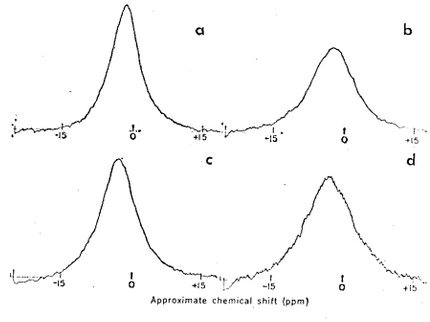

Fig. 1. Representative spectra are: (a) lysozyme; (b) ovalbumin; (c) native serum albumin (*p*H 5.03); and (d) *p*H "denatured" serum albumin (*p*H 2.45). Experimental conditions were: 60 Mhz NMR, −35°C, 0.001*M* KCl, all solutions approximately 75 mg/ml of protein except (d) which was 50 mg/ml but whose spectrum was obtained at twice the spectrum amplitude of the others. All spectra are averages of four repetitions, using the C-1024 signal averager.

An important problem is created by the high pressures generated when the frozen preparations warm. There is a real risk of cracking the sample tube and, possibly, the Dewar insert. We routinely used coaxial tubes made from a standard 5-mm tube and an inner tube made from ordinary 4-mm stock pyrex. Risk to the insert is greatly reduced if the space between the tubes is kept dry and if the samples are allowed to warm to probe temperature outside the spectrometer.

Linewidths are reported as widths at half-height of the absorption curves. Relative amounts of hydration were obtained from the area measured as the product of linewidth and height (*1*). The actual bores of the 4-mm sample tubes were measured and results are corrected to a 2.5-mm inner diameter. Absolute hydration was estimated by comparison with a standard water sample containing 0.01*M* Mn^{++} and 4.5*M* LiCl. At −35°C, this sample was unfrozen, had a linewidth of 250 hz, and a water concentration of 50 ± 1*M* (*2*). We found that the linewidths of the narrower signals could be measured to ± 20 hz. Areas on such lines could be determined to ± 10 percent. Weak, broad signals were much more difficult to analyze, with errors approaching 50 percent for the weakest signals.

The bound water signal (Fig. 1 and Table 1) is directly proportional to the concentration of the macromolecule. Significant variations in both linewidth and relative hydration are observed. A tRNA sample is included for purposes of comparison. The water signal has some sensitivity to conformational perturbations, such as thermal denaturation (protein solutions were boiled until coagulation occurred) and the acid expansion of serum albumin (*3*, p. 517). Ionic strength also alters the signal. In the absence of salt, a relatively broad line is observed which narrows approximately twofold at moderate ionic strength (0.1*M*) and then broadens again at salt concentrations greater than about 1*M*. These effects have been observed with KCl, NaCl, and $(NH_4)_2SO_4$. The chemical shift of the bound water did not depend on the macromolecular solute within the uncertainties set by the linewidths. On freezing, a small upfield shift (0.5 part per million) was universally observed. Both liquid and bound water signals move progres-

Table 1. Protein hydration at −35°C and 60 Mhz.

Sample	Normalized conc. (mg/ml)	Linewidth (hz)	Signal height (arbitrary units)	Hydration* (g H_2O per g of protein)
Gelatin in distilled water	100	800	5.2	0.4-0.5
	50	900	2.9	.4- .5
	25	(900)	1.4	.4- .5
Lysozyme	100	400	11	.36
α-Chymotrypsin	100	460	9	.37
Bovine serum albumin	100	480	11	.43
Oxyhemoglobin	100	480	11.5	.45
Ovalbumin	100	620	8	.31
tRNA, denatured †	100	250	140	1.7
α-Chymotrypsin, denatured	100	630	7.5	0.4
Ovalbumin, denatured	100	880	5.5	.3
Bovine serum albumin ‡				
*p*H 4.5-5.3	100	510	11.2	.37
3.4-4.0	100	580	8.2	.31
2.0-3.0	100	670	6.4	.28

* Absolute values may be uncertain by ± 20 percent; relative values are more reproducible (± 10 percent or better). † Hendley *et al.* (*17*). ‡ Averaged values.

sively downfield as the temperature is lowered.

Temperature effects on linewidths and areas are readily summarized. Linewidths increased exponentially as the temperature dropped, with an Arrhenius activation energy of 4 to 5 kcal/mole for all protein solutions. Linewidths within a few degrees of the melting point of the bulk solvent deviated from the Arrhenius plot. The area under the absorption curves decreased dramatically on passing through the freezing or eutectic point of the bulk solvent and then remained constant (± 10 percent) for 20°C. Some loss of signal at lower temperatures might be a real effect or might be due to a systematic error in integration of the broad lines. No freezing point of the bound water was detected over the temperature range studied (to −50°C).

Unfrozen liquid due to severe thermal gradients or lack of thermal equilibrium appears unlikely from control experiments on pure water and aqueous solutions of NaCl. Freezing points were within a few degrees of the accepted values, and the width of the phase transitions indicated a thermal gradient of less than 10°C. No signals were observed after the transition was complete. Contributions to the signal from exchangeable protons of the solute are estimated at not more than 10 percent (*3*, p. 554) even if the exchange proceeds at these low temperatures. Finally, our linewidths and activation energies are in good agreement with those reported by Brey *et al.* (*4*), who studied small amounts of water adsorbed on lysozyme and bovine serum albumin.

If we assume we are observing bound water, some conclusions can be drawn from our data. First, we can compare our results with those from other methods of determining hydration. General agreement is reasonable when it is remembered that the hydrodynamic determinations shown are maximum estimates based on the assumption of a spherical protein molecule (*3*, sections 21 and 23; *5*). Conversely the vapor equilibrium experiments might be expected to set a lower limit to the degree of hydration found in solution. There is good agreement with the work of Anderegg *et al.* (*6*). Their result is based on a measured axial ratio (low-angle x-ray scattering) and experimental frictional coefficient and viscosity data. Similar agreement with one of the possible axial ratios for lysozyme (*7*) is also shown. These findings support the utility of the Oncley "contour" diagrams (*8*) and indicate the possibility of determining axial ratios from direct hydration measurements. The high degree of hydration of RNA (Table 1) is also consistent with the known hydrophilicity of nucleic acids from x-ray data (*9*).

Second, the detection of such relatively narrow NMR signals well below the normal freezing point of water is evidence that the bound water is not literally "ice-like." Further, the lack of a definite freezing point for the bound water indicates that it is not in a well-organized structure, even though it is more restricted in mobility than liquid water. Extrapolation of the data in Table 1 suggests that the confinement persists at room temperature, in agreement with relaxation studies (*10*). The activation energies for proton mobility were 4 to 5 kcal/mole, a value consistent with a single hydrogen bond barrier (*4*). By comparison the activation barrier in ice is 12 kcal/mole (*11*). If there exists a significant amount of bound water that is well-organized and ice-like, one must argue that the "icebergs" exchange protons or water molecules rapidly with other, more mobile, bound water (unlike ice), or, alternatively, that the icebergs are there but are not observed. The adsorption experiments (*4*) suggest that less than 0.05 g of H_2O per gram of protein escapes detection. Thus, there appears little margin for the existence of ice-like bound water.

Another conclusion is that the conformational changes have been accompanied by a change in the type or degree of hydration or both. An example of the qualitative information that this technique can yield is the finding that the marked insolubility of thermally denatured globular proteins is not caused by extensive dehydration (Table 1).

The most interesting questions center around the nature of the bound water. Our present hypothesis is that the water detected in the NMR experiment is prevented from joining the normal ice lattice. We can imagine two classes of water that might not freeze easily. First, water physically or chemically adsorbed on surfaces (*12*) has a lowered freezing point. A similar effect could exist in frozen solutions of macromolecules where a thin film

Table 2. Hydration of proteins by various methods. Results are expressed as grams of H_2O per gram of protein.

Protein	Present work	Hydrodynamics (max. hydration)	Vapor equilibrium	Other*
Ovalbumin	0.31	0.45†	.30‡	
Lysozyme	.36	.89†	.25‡	0.33–.35§
Chymotrypsin	.37		.25–.30‖	
Serum albumin	.43	1.07†; 0.75¶	.32‡	.40#; .48#
Hemoglobin	.45	0.36†; 0.69¶	.25–.30‖	

* For a summary of other results, see Fisher (*18*). † Tanford (*3*, p. 359) (frictional coefficients). ‡ Bull (*19*). § Calculated with Oncley's diagrams (*8*) and the following data: axial ratio, 2.2 (*7*); intrinsic viscosity, 2.98 cm^3/g (*7*); specific volume, 0.688 cm^3/g (*3*); and frictional coefficient, 1.210 (*20*). ‖ McMeekin *et al.* (*21*). ¶ Tanford (*3*, p. 395) (intrinsic viscosities). # Anderegg *et al.* (*6*). The first value is calculated from viscosity data and the second from frictional coefficient.

(perhaps a monolayer) of water separates the solute from the ice. This layer might reflect the shape of the macromolecule. Such water could be held by hydrogen bonds, or it might be in a region where the normal ice structure was disrupted through "hydrophobic" interactions (*13*). Another source of unfrozen water could exist if the macromolecule was able to actually trap water in sufficiently small channels or pores (*12*). One might anticipate a relatively sharper resonance line for water of this type because, other things being equal, water in a cavity would have lower activation energy than water on a surface (*14*). In a molecule with both types of water, considerable averaging would be expected (*15*).

Approximate combinations of "adsorbed" and "trapped" water can be used to explain the protein results on an ad hoc basis. Assume the native protein contained some of both types of water. Denaturation of the proteins could leave the amount of surface binding approximately unaltered, or even augmented, as interior sections of the molecule were exposed. However, the water held inside the native structure would become accessible as the molecule opened, losing any special character or mobility. If the "internal" water is associated with a narrow signal, the line broadening on denaturation can be understood. The simplest description of the water detected by the NMR technique would seem to be water that is constrained but has no definite "long-range" organization.

Our method is not limited to either water as a solvent or biomacromolecules as solutes. Any system in which the solute has a moderate-to-high molecular weight and the solvent has a moderately high freezing point might well yield similar results.

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References and Notes

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Cytoplasmic DNA from Petite Colonies of Saccharomyces cerevisiae: A Hypothesis on the Nature of the Mutation

Abstract. *The density of the cytoplasmic DNA of two strains of "petite" mutants of yeast, obtained by treatment with acriflavin and with ultraviolet light, was examined in cesium chloride density-gradient centrifugation and in all cases appeared to be less than that of the wild type. A cytoplasmic respiratory-deficient strain, treated with additional acriflavin, can show a further shift of the position of the satellite band, always in the direction of reduction of density. Also, from the $\rho^+ \times \rho^-$ cross, ρ^- strains can be recovered in which the density of the satellite DNA is different from the density of the parent ρ^- strain. This finding suggests the existence of recombination in cytoplasmic DNA molecules.*

In yeast the enzymes linked to the respiratory processes, with the single exception of cytochrome c, are under double genetic control [for a concise review of the problem, see Mounolou *et al.* (*1*)]. The cytoplasmic mutation, which leads to the formation of petite colonies, in addition to having an exceptionally high spontaneous frequency of mutation (*2*), can also be induced with a frequency near 100 percent by acriflavin (*3*) and other agents, such as fluorouracil (*4*). Therefore, the mutation has been thought to consist of the elimination of cytoplasmic genetic determinants from the cells (*5*). Discovery of the phenomenon of "suppressiveness" (*6*), that is, that the mutant can be dominant with respect to the wild type, has lead to the belief that the cytoplasmic determinant was still present, although altered, in the mutated cells. Further, Carnevali and Tecce (*7*), Carnevali *et al.* (*8*), and Mounolou *et al.* (*9*) noted that the ρ^- strains contain a band of satellite DNA, corresponding to mitochondrial DNA (*9*), having a density different from that of the DNA of the wild type ρ^+ strain. In particular, Carnevali *et al.* (*8*) found that the density of the satellite band was 1.671 g/cm^3 as compared to 1.686 g/cm^3 for the DNA from the ρ^+ strain (*10*). The density of 1.671 g/cm^3 corresponds to the density of a polymer that contains mainly deoxyadenylate and deoxythymidylate. In a later work (*11*) it has been confirmed that the DNA from the mutant strain is composed almost exclusively of adenine and thymine. We now report data that, together with previous data, permit the formulation of a hypothesis on the mechanism of the mutation.

We used strain 42 ρ^+ diploids that were heterozygous for some nutritional markers and strain 34, derived from the former by means of spontaneous somatic recombination and homozygous for arginine and methionine requirements. We also used strain 16 ρ^+.

Cytoplasmic mutations were obtained by treatment with acriflavin and with ultraviolet light. The treatment with acriflavin was carried out with 0.005 percent (weight to volume) acriflavin,