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WATER RELATIONSHIPS IN FOODS

Advances in the 1980s and
Trends for the 1990s

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PROTEIN-WATER INTERACTIONS FROM ^2H NMR RELAXATION STUDIES: INFLUENCE OF
HYDROPHILIC, HYDROPHOBIC, AND ELECTROSTATIC INTERACTIONS

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ABSTRACT

The importance of water interactions with proteins in food systems is well documented. A controversy exists, however, as to the nature of these interactions and the effect of protein structural changes on them. To clarify these questions, a method has been developed for determining hydration from the protein concentration-dependence of deuteron resonance relaxation rates. Measurements were made in D_2O on β -lactoglobulin A to study effects of hydrophilic interactions, and on both casein micelles and submicelles to study hydrophobic and electrostatic effects. From the protein concentration-dependent relaxation rates, the second virial coefficients of the proteins were obtained by nonlinear regression analysis. Using either an isotropic tumbling or an intermediate asymmetry model, hydrations, \bar{v} , and correlation times, τ_c , were calculated for the protein-associated water; from τ_c , the Stokes radius, R , was obtained. Variations in \bar{v} and R were in accord with known structural changes in molecular states of the proteins. The NMR results are compared with hydrations and structural information derived independently from small-angle X-ray scattering.

GENERAL INTRODUCTION

The importance of water interactions with naturally occurring biological macromolecules is well documented.¹ It is generally considered that the quantitation of this interaction will aid in enabling investigators to understand the energetics of protein secondary, tertiary, and quaternary structure as well as changes in the relevant global structural parameters with varying environmental conditions. However, to date, not even the amount of water "bound" (i.e. of reduced mobility) to a globular protein (hydration) can be determined with confidence, much less can one directly observe the constituent groups on the protein responsible for the "binding" of water. To further complicate the problem, the hydration values of proteins have been found to be dependent on the methodology used in the study. Traditionally, hydrodynamic methods, i.e. sedimentation, translational and rotational diffusion, and viscosity, have been used to measure the hydration of proteins with some success; with these, however, an assumption of the overall shape of the unhydrated protein must be made.²⁻⁴

In lieu of hydrodynamic data, it will be shown in this chapter that NMR

relaxation techniques are capable of giving information on hydration. While the absolute values of NMR hydration are not completely independent of assumptions regarding relaxation mechanism, relative values are largely independent of such assumptions and can serve to assess changes in structure or biochemical behavior as a function of varying environmental conditions. The interpretation of such changes, of course, will depend on the system studied.

One way of using NMR relaxation to obtain this kind of information has been by means of frequency dependence.⁵ There are, however, few instruments available on which dispersive measurements can be made. Attention must also be paid to the frequencies chosen: if measurements are made at two different frequencies to eliminate the use of a second mode of relaxation, care must be taken that the frequencies are sufficiently different to exhibit a substantial-enough dispersion for adequate relaxation statistics. In any case, the frequencies must be high enough (and so must the molecular weight, on which the correlation time depends) to escape the line-narrowing region, i.e. the product $\omega_0^2 \tau_c^2$ (where ω_0 is the Larmor frequency and τ_c the correlation time) must be clearly larger than 1. Otherwise, the system of requisite simultaneous equations will either have no solution, or the solution will be very imprecise.

In place of frequency dependence, concentration dependence (readily accessible with any modern NMR instrument) may be used to advantage. Two modes of relaxation, longitudinal and transverse, have been used with deuterium resonance to avoid the cross relaxation encountered with spin-lattice relaxation of protons. Relaxation of ^{17}O has also been used, for similar reasons.^{6,7} In a different method, proton spin-lattice relaxation was employed, but cross-relaxation effects were determined and allowed for by the joint examination of two genetic variants of the same protein, which differed only in the known extent of an association reaction.⁸ Again, the combination of protein molecular weight and resonant frequency must satisfy the condition for avoiding the line-narrowing mentioned.

In addition to hydration, NMR relaxation can give other valuable information that can be correlated with structural and biochemical changes. Correlation times are related to molecular size and shape and are relevant to hydrodynamics. Virial coefficients, for which concentration-dependence data are indispensable, can be calculated directly from a concentration plot. It is true for either mode of relaxation that the observed relaxation rates are linear functions of hydration, concentration, and an exponential containing both the concentration and the virial coefficient, B_0 . Nonlinear regression, applied to this relationship, will give B_0 directly. Virial coefficients, related to the average net charge carried by molecules, provide a particularly useful measure of molecular interaction. All of these parameters can in turn be related to known structural states of biomacromolecules. Correlations between changes in structural state and NMR-derived parameters give new insights into the dynamic interactions of a system.

Water and NMR Relaxation: The individual molecules of water, although constrained by water structure, as discussed in other chapters, are in a dynamic state and show very rapid movements such as translation and rotation. It is important to keep these properties in mind for the following discussion. A water molecule can rotate about its own axis once every 3 picoseconds; in addition, it can exchange position with another molecule in the water matrix, on a scale of femtoseconds. If we impose a properly selected external magnetic field, these dynamic properties are not interfered with, but the spinning nuclei of the water protons will begin to align their magnetic moments with this field.¹⁻⁵ At thermal equilibrium, a majority of the magnetic moments of these nuclei will be aligned with the field. By putting energy into the system in the form of pulsed radio frequencies, we can force these nuclei out of alignment (more energy) in specified directions. When

the pulse is ended, we can measure the rates at which the nuclei return to their original (less energetic) states. Their return to the original (ground) state is modulated in part by the translational and rotational motions of the excited water molecules; in a sense, they give up their energy through their motions. The radio-frequency pulse sequences selected, as described in Methods and Materials, result in relaxation in two modes: R_1 , or longitudinal relaxation, and R_2 , or transverse relaxation. These rates of relaxation of the water molecules as a whole are measured in these experiments.

Proteins and Water Relaxation: If we introduce proteins into the water and then conduct the relaxation experiments, the relaxation rates (both R_1 and R_2) tend to increase as a function of added protein concentration.^{8,9} The reasons for this phenomenon are quite complex, and the theory and testing of the hypotheses connected to this have been demonstrated and reviewed in detail elsewhere.^{8,9} Briefly, an explanation is as follows:

1 - water "bound" to the protein has a different rate of relaxation than "free" (i.e. of unrestricted mobility) water; the "bound" water moves essentially with the more slowly rotating protein (e.g. on a scale of nanoseconds for β -lactoglobulin).

2 - "free" water and "bound" water exchange rapidly, so that excited "free" water can "bind" to the protein, give up its energy (relax), and rapidly return to the solution, and vice versa.

Other mechanisms of relaxation can occur and can cloud the issue of whether or not NMR relaxation measurements can measure "bound" water. Perhaps the most serious consideration of these is the phenomenon of cross-relaxation. Since this occurs in R_1 measurements of protons but not deuterons, we shall discuss only $^2\text{H-NMR}$ R_1 and R_2 relaxations of the proteins. Other complications, such as whether one needs to consider two or three "states" of water (that is, "bound" vs. "free", or "bound" vs. "free" vs. protein-influenced) have also been reviewed.^{8,9} For this chapter, we will concentrate only on a simple two-"state" model.

INFLUENCE OF PROTEIN HYDROPHILIC SELF-ASSOCIATION ON WATER-PROTEIN INTERACTIONS: β -LACTOGLOBULIN A

Previously derived relationships^{8,9} were tested on bovine β -lactoglobulin A (β -Lg A), the major whey protein in milk, under various environmental conditions. At pH 5.2, β -Lg A exists as a dimer of molecular weight 36,200. At pH 4.65, β -Lg A undergoes a dimer-to-octamer self-association involving hydrophilic groups. ^2H NMR relaxation measurements made at the above conditions served to test whether the notion of increased hydration with increasing hydrophilic self-association is valid.

Experimental Procedures

Preparation of Solutions: The following procedures are described to illustrate methods that have led to satisfactory results in studies of β -Lg in solution.⁹ Protein solutions to be used for proton resonance measurements, prepared 1 day before use, were exhaustively dialyzed overnight against buffer at 0-5°C; dilutions for the concentration series to be studied were made with the appropriate dialyzate. Solutions to be used for deuterium resonance measurements were made up from a stock solution prepared by partial deuterium exchange. A suitable amount of crystalline protein in a stoppered vial was allowed to equilibrate repeatedly for 24-h periods at 4°C as a slurry with a small quantity of D_2O , followed by high-speed centrifugation and addition of fresh D_2O , for a total of five times. The solutions were buffered by direct addition of solid potassium phosphate, and the pH was adjusted by addition of 0.1 N NaOD in D_2O . Concentrations of β -Lg were determined spectro-

photometrically from an absorption coefficient of $0.96 \text{ ml} \cdot \text{mg}^{-1} \cdot \text{cm}^{-1}$ at 278 nm.¹⁰

Relaxation Measurements: Deuteron NMR spectra were obtained by Fourier Transform spectroscopy with a JEOL FX60Q spectrometer operating at a nominal proton frequency of 60 MHz. The frequency of observation was 9.17 MHz. Raw data were in the form of relative intensities as calculated by the JEOL 980B computer.

Since the high concentration of water (D_2O) in a dilute solution produces an intense signal, a single accumulation at the particular sample temperature (2° , 15° , or $30^\circ \pm 1^\circ\text{C}$) was sufficient for each spectrum. Even then, care was necessary to avoid exceeding the dynamic range of the computer with consequent truncation. To this end, small sample volumes were employed by use of a microcell assembly with an expendable $35\text{-}\mu\text{l}$ sample bulb, available from Wilmad Glass Co., Inc. The protein solution was introduced very slowly into the spherical bulb by means of a fine-gauge syringe needle inserted through its capillary neck, to avoid the inclusion of any air bubbles. Bubbles trapped below the neck could lead to vortex formation in the spinning sample bulb, vitiating the necessary assumption of a spherical sample geometry. The bulb, suspended by its neck from a chuck attached to a plastic cap, was positioned snugly inside a precision 5-mm O.D. sample tube which, initially, contained also the lock-signal solvent. The small amount of this solvent in the residual annular space outside the bulb was not always sufficient to assure maintenance of the lock; occasional failure of the lock during a lengthy series of automatic measurements resulted in loss of usable data. A second arrangement was then used in which the 5-mm tube, containing the sample bulb but no solvent, was positioned by means of fluorocarbon plastic spacers concentrically within a precision 10-mm O.D. sample tube accommodating a much larger quantity of lock-signal solvent. Incidental advantages of this arrangement were that the outside of the sample bulb was thus kept dry, and that the solvent could be sealed within the annular space between the two tubes and so kept from contamination for a greatly extended time. Except for these advantages, either arrangement resulted in the same measurements. The cell assembly, in either case, was positioned in the JEOL FX600 10-mm $^1\text{H}/^{13}\text{C}$ dual-probe insert.

Longitudinal relaxation rates, R_1 , were measured by the inversion-recovery method, where the repetition time, T , in the pulse sequence [...T... π ... τ ... $\pi/2$...] was chosen to be at least five times T_1 ($= R_1^{-1}$), and the values of the variable delay time, τ , ranged from 10 ms to 3s, for a total of between 5 and 20 τ values, depending on the detail desired. Under the conditions of this method, the relation of the peak intensity, A_τ , to the pulse delay time, τ , becomes

$$A_\tau = A_\infty [1 - 2 \exp(-R_1\tau)], \quad [1]$$

where A_∞ is the limiting peak intensity for $\tau \rightarrow \infty$. Independent measurement of A_∞ , a source of irreducible error, can be dispensed with, and the problem of weighting the data points in the conventional linear plot of the logarithm of a function of relative peak heights can be eliminated, by fitting directly to the data points (τ , A_τ), by least squares, an exponential of the form of Equation [1], from which the two parameters, A_∞ and R_1 , can be obtained (Fig. 1, curve A). The fitting of this two-parameter exponential was carried out by computer by means of an iterative program. For each sample, R_1 was determined at least four times, and the results were averaged; standard errors amounted to 1-2%. This procedure was repeated at each concentration; a minimum of six concentrations were used under each set of conditions of temperature at which resonance relaxation was examined.

Transverse relaxation rates, R_2 , were determined by spin-locking measurement¹¹ of $R_{1\rho}$, the longitudinal relaxation rate in the rotating frame. R_1 ,

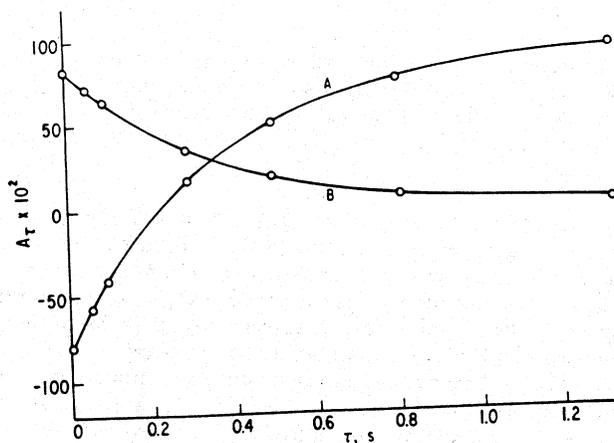


Fig. 1. Deuteron resonance peak intensities. A_τ , as a function of time, τ , for casein micelle solution at pH 6.75, 15°C, in D_2O (78.5 mg/ml) and in PIPES-KCl- $CaCl_2$ buffer. A. Longitudinal relaxation measurements (R_1) from inversion-recovery method. B. Transverse relaxation measurements (R_2) from spin-locking measurements of $T_{1\rho}$. Solid lines are best fit (F-test) of corresponding equations in text (Equation [1] and [2]) to experimental intensities by nonlinear regression.

equals R_2 in dilute solutions of low viscosity whenever the magnitude of $R_{1\rho}$ is independent of $H_{1\rho}$, the spin-locking radio-frequency field in the rotating frame;¹¹ this was the case, within the limits of experimental error, in the present work. R_2 was evaluated as described above for R_1 , except that the relation between peak intensity, A_τ , and decay time, τ , in this case becomes

$$A_\tau = A_0 \exp(-R_2\tau), \quad [2]$$

where the initial intensity, A_0 , replaces A_∞ as the maximum peak intensity. Again, a least-squares two-parameter exponential fit to the data points was performed by an iterative computer program, from which A_0 and R_2 were obtained (Fig. 1, curve B).

For each sample, R_2 was determined with the same number of replications as R_1 ; standard errors amounted to 2-3%. Measurements of one mode of relaxation were made on the identical sample and immediately following the completion of measurements of the other mode, or at the latest, the next day. Measurements for deuteron relaxation at 9.17 MHz were made at pH 7.0 and at 2°, 15°, and 30°C ± 1°C, respectively. These rates were measured in D_2O to eliminate cross-relaxation effects between water and protein protons, such as observed by Edzes and Samulski¹² and Koenig et al.¹³ Residual H_2O in the sample will have little effect on the D_2O rates, since even at elevated (1:1) ratios, D_2O relaxation is not compromised by the presence of H_2O .¹³ However, in samples prepared as described above, the residual H_2O is always < 5%.

Analysis of Data

Protein Activity Model: For a two-"state" model ("bound" and "free" water), Kumosinski and Pessen⁴ have shown that for the change in R_{obs} , the observed longitudinal or transverse relaxation rate of water in the presence of varying protein concentration, c ,

$$R_{\text{obs}} - R_f = (R_b - R_f) \bar{\nu}_w a_p/W,$$

where R_f is the appropriate relaxation rate of "free" water (R_1 or R_2), R_b is the corresponding relaxation rate of "bound" water, W is the total concentration of water, and a_p is the activity of the protein. $\bar{\nu}_w$ is the degree of hydration (i.e. basically, the average number of molecules of water "bound" per molecule of dry protein or, in units consistent with the concentration units employed, the number of grams of "bound" water per gram of dry protein). For ligands in general, $\bar{\nu}_w$ differs from n , the number of available binding sites per substrate molecule, the difference being a function of association constant and ligand concentration. In the case of water, however, which is a ligand present in such vast excess that the substrate is saturated with it, the distinction between $\bar{\nu}_w$ and n disappears. In the following, we will, for simplicity and convenience, use the expression "hydration" for short to indicate the quantity $\bar{\nu}_w$ in units of g/g. Also,

$$a_p = c \exp(2B_0c + \dots),$$

where B_0 is the second virial coefficient of the protein.

Data points of the observed relaxation rate (longitudinal or transverse) vs. protein concentration were fitted with a combined function of Equations [3] and [4] via an iterative Gauss-Newton nonlinear regression program developed at this laboratory. Analysis by this program produced values for B_0 , $[(R_b - R_f) \bar{\nu}_w]$, and R_f . Values for R_{1b} or R_{2b} , $\bar{\nu}_w$, and τ_c were obtained by simultaneous solution of the Kubo-Tomita-Solomon equations,^{14,15}

$$R_{1b} = 2K\tau_c[(1 + \omega_0^2\tau_c^2)^{-1} + 4(1 + 4\omega_0^2\tau_c^2)^{-1}]$$

and

$$R_{2b} = K\tau_c[3 + 5(1 + \omega_0^2\tau_c^2)^{-1} + 2(1 + 4\omega_0^2\tau_c^2)^{-1}],$$

where R_{1b} and R_{2b} are the longitudinal and transverse relaxation rates, respectively, and τ_c is the correlation time of the "bound" water; ν_0 (or $\omega_0 = 2\pi\nu_0$) is the nuclear angular precession frequency (Larmor frequency) in Hz or in radians per second, respectively; and K is a measure of the strength of the nuclear interaction, i.e.

$$K = (3/80)(e^2qQ/\hbar)^2(\eta^2/3 + 1)S^2.$$

Here, e is the electronic charge, 1.6022×10^{-19} coulomb, q is the electric field gradient, Q is the nuclear electric quadrupole moment, \hbar is Planck's constant divided by 2π , 1.056×10^{-27} erg*s, η is a dimensionless parameter measuring the deviation from axial symmetry,¹⁶ and S is the order parameter for intermediate asymmetry of the motion of the "bound" water.⁷ Hence, this thermodynamic theory can be used whether isotropic ($S = 1$) or anisotropic motion ($S + 1$) is hypothesized, where in the latter case, the "bound water" should be thought of in the sense of "hydrodynamically influenced layers" or "surface-induced probability distribution of water molecules." For these experiments, η is assumed to be zero; $\nu_0 = 9.17$ MHz and $e^2qQ/\hbar = 215.6$ kHz.¹⁷

Proof of Activity Model and the Multicomponent Expression: At this point, an observation regarding predictions resulting from the derived multicomponent expressions above may be in order. Since these are based ultimately on equilibrium constants, the mass terms should be properly expressed as activities instead of concentrations. Consequently, relaxation rate vs concentration curves should be expected to be nonlinear whenever there are appreciable protein activity coefficients. The concentration dependences of R_1 and R_2 for β -Lg A at pH 6.2 and 30°C, and of R_2 at pH 4.65 and 2°C, were studied. Under these conditions, the charge-to-mass ratio and the second virial coefficient are relatively small ($B_0 = 0.9$ ml/g).¹⁰ In fact, all these

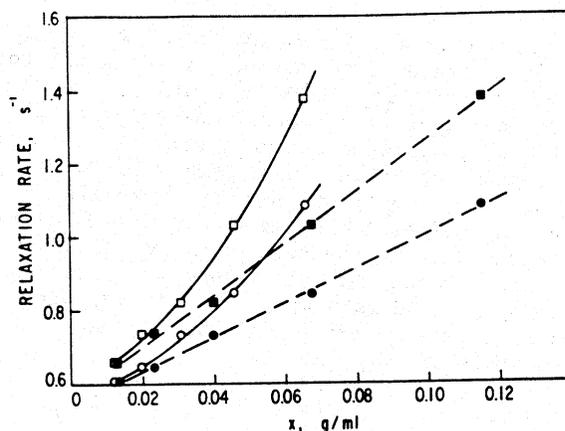


Fig. 2. Dependence of water proton relaxation rates of β -Lg A on both concentration and activity, at pH 2.7 and 10°C. Transverse relaxation rates (squares) as function of X (-□-□-: X = concentration; --□--□--: X = activity). Longitudinal relaxation rates (circles) as function of X (-o-o-: X = concentration; --o--o--: X = activity). All points represent experimental values; lines represent least-squares polynomial fits of highest degree to make a statistically significant contribution to goodness of fit (F-test). Dependence on concentration is found to be of second degree as consequence of charge effects at pH 2.7. (Polynomial coefficients for R_1 , $a_0 = 0.562$, $a_1 = 2.926$, $a_2 = 75.86$; for R_2 , $a_0 = 0.606$, $a_1 = 3.468$, $a_2 = 125.9$.) Activities take these effects into account, and dependence on activities is linear. (Coefficients of straight line: for R_1 , $a_0 = 0.541$, $a_1 = 4.691$; for R_2 , $a_0 = 0.560$, $a_1 = 7.061$). (Taken from ref. 9.)

data exhibit linear relationships over a concentration range from 0 to 0.08 g protein/g water, where γ does not differ greatly from unity. Fig. 2, on the other hand, shows corresponding plots for pH 2.7 and 10°C, where the net charge is approximately 40 and the second virial coefficient is 8.5 ml/g.¹⁸ The plots in terms of concentration here are clearly nonlinear. A change in the concentration scale to g protein/g water had no significant effect on the nonlinearity of the plots. (Low-temperature data were used at this pH for experimental reasons: the protein under these conditions undergoes a dissociation from dimer to monomer, but at low temperature, the amount of monomer at concentrations in excess of 0.01 g/ml is negligible.¹⁸)

A polynomial curve-fitting program was used with all data in Fig. 2 as well as all other concentration-dependent data in order to determine linearity or nonlinearity, the degree of the polynomial being determined by goodness of fit as judged by the standard F-test. (The regression program used selects the lowest-degree expression for which the sum of squares due to the addition of one higher degree is statistically insignificant.) The two concentration

Table 1. Deuteron NMR Relaxation and Hydrodynamic Results for β -Lactoglobulin in Solution^a

pH	T, °C	R_{1f} , s ⁻¹	R_{2f} , s ⁻¹	τ_c , ns	r_{NMR}^b , nm	r_{sed} , nm	$(\tau_c)_{calc}$, ns
6.2	30	1.93 ±0.01	1.93 ±0.04	10.0 ±2.7	2.32 ±0.19	2.70	10.2
	2	5.46 ±0.24	5.25 ±0.28	25.6 ±4.1			22.5
4.65	30	2.01 ±0.04	1.79 ±0.12	22.5 ±3.3	3.04 ±0.15	4.35	65.9
	2	4.90 ±0.16	4.67 ±0.44	32.2 ±4.6			145.0

^a Adapted from ref. 9.

^b Error terms in this and subsequent tables represent the standard error of the parameter.

^c Spherical model assumption.

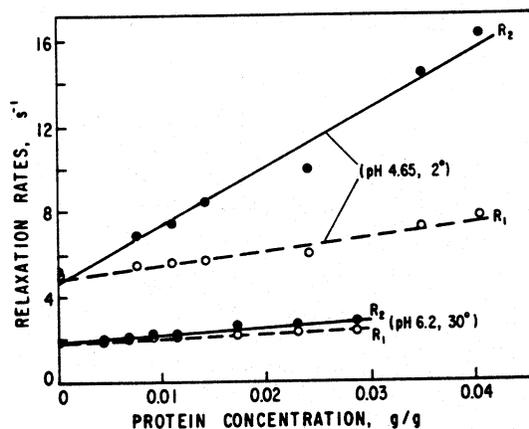


Fig. 3. Dependence of deuteron relaxation rates on β -Lg A concentrations (g protein/g water) in D_2O . Transverse relaxation rates, R_2 (—●—), at pH 4.65, 2°C and at pH 6.2, 30°C. Longitudinal relaxation rates, R_1 (---○---), under the same two sets of conditions. Points represent experimental values; lines represent least-square fits. Points at all concentrations show linear relationship of relaxation rates to concentration, at both sets of pH and temperature conditions, and for both modes of relaxation. (Taken from ref. 9.)

plots of Fig. 2 give polynomials of degree two. Protein concentrations, c , were then transformed into activities, a , by means of the relationship $a = \gamma c$, where the activity coefficient, $\gamma = \exp(2B_0 c)$, was obtained (see Eq. [4])

from the second virial coefficient, $B_0 = 8.5$ ml/g, as cited above. Activity plots corresponding to the concentration plots are also shown in Fig. 2; it is evident that these are linear by the same criteria.

Under the conditions of Fig. 2, γ is sensibly larger than unity and, even in the presence of salt, failure to treat R as a function of activity rather than concentration will evidently lead to excessively high values of hydration. The opposite would be true under conditions when, in the absence of salt, charge fluctuations and consequent intermolecular attraction exist. With γ less than unity, a concentration plot in place of the correct activity plot for R must lead to inordinately low values of hydration. Alternatively, protein concentrations could continue to be used, provided the relevant terms are multiplied by $(da/dc)_\mu$, the change in activity with concentration at constant chemical potential.

These results may be taken to demonstrate the validity of the multicomponent expression. This expression is used in the following in analyzing the data to describe the hydration of the genetic A variant of β -lactoglobulin, β -Lg A, under nonaggregation conditions, as well as under the well-characterized dimer-to-octamer association.¹⁸⁻²² (This phenomenon, because it involves a 4-fold association of dimer under conditions where dissociation to monomer is negligible, is referred to in the following as "tetramerization.") It has been demonstrated that here the virial coefficients are small,^{10,18} so that it is permissible to simplify the treatment by using protein concentrations.

Isotropic "Binding," Two-"State" Model

Determination of Correlation Times: Concentration plots of R_1 and R_2 for D_2O (Fig. 3) showed no evidence of nonlinearity, at either pH and either temperature, over the concentration range studied. This agrees with the low virial coefficient of β -Lg A under these conditions.¹⁰ The relaxation increments, k_1 and k_2 , together with the corresponding intercepts, R_{1f} and R_{2f} (Fig. 3), were used to determine the "bound"-water correlation times, τ_c , shown in Table 1. As can be seen, τ_c increased as the temperature decreased; this is in quantitative agreement with the requirement of Stokes' equation that τ_c increase with both increasing viscosity and decreasing temperature. Furthermore, τ_c also increased when the pH was lowered from 6.2 to 4.65, as would be predicted from the experiments of Timasheff and co-workers.¹⁸⁻²²

Determination of Hydration Parameters: The deuterium NMR relaxation increments were used, with a value of $e^2qQ/\hbar = 215.6$ kHz¹⁷ and with the asymmetry parameter, η , assumed to be zero. This type of calculation gives low values of \bar{v}_w , as shown in Table 2; however, low values could be expected, because the relaxation increment probably samples only a percentage of the total hydration of a protein, since at 9.17 MHz, any "bound" water with τ_c values less than 6 ns would have a T_1/T_2 ratio of unity. At pH 4.65, where tetramerization occurs, the hydration markedly increases with decreasing temperature, whereas at pH 6.2, where none occurs, the hydration is lower and independent of temperature. This is consistent with the findings of Timasheff and co-workers^{19,23} from small-angle X-ray scattering that the geometry of the octamer must include a large central cavity in which trapped water could reside.

Comparison of Results with Other Structural Information: Dynamics of β -Lg Dimer. With the τ_c values calculated from k_2/k_1 from deuterium NMR spin and spin-lattice relaxation increments, dR_2/dc and dR_1/dc , a Stokes radius, r_{NMR} , for the "bound" water can be calculated from the Stokes-Einstein relation²⁴ on the basis of a spherical model (Table 1). At pH 6.2, where β -Lg A exists as the unassociated dimer, the value of r_{NMR} is slightly lower than the one for the protein itself derived from hydrodynamic data, r_{sed} .²⁵ This discrepancy could be due to the spherical approximation inherent in the use of the Stokes-Einstein equation, inasmuch as the β -Lg dimer actually has

Table 2. Hydration and Thermodynamics for β -Lactoglobulin from Deuteron NMR Using $(e^2qQ/h) = 215.6$ kHz^{a,b}

pH	T, °C	$\bar{\nu}_w$, g H ₂ O/g protein	ΔG , kcal	$-\Delta H$, kcal	$-\Delta S$, e.u.
6.2	30	0.0063 ± 0.0008	0.90 ± 0.08		
	2	0.0072 ± 0.0020	0.74 ± 0.15	0.8 ± 3.0	6 ± 10
4.65	30	0.0095 ± 0.0002	0.65 ± 0.01		
	2	0.0301 ± 0.0003	-0.044 ± 0.006	6.9 ± 1.6	24.8 ± 5.8

^aAdapted from ref. 9.

^bMethod I, ref. 9.

an axial ratio of approximately 2:1.¹⁹ Moreover, the Stokes radius of the protein obtained from sedimentation includes the water of hydration and should therefore be larger than the Stokes radius of the "bound" water calculated from the ²H NMR relaxation data. What should be compared with the τ_c of the "bound" water is the τ_c of the protein without any contribution from hydration. For the latter, values of 10.2 ns at 30°C and 22.5 ns at 2°C (Table 1) can be calculated for the protein with the use of its partial specific volume, 0.751 ml/g, and an asymmetry factor of 1.168²⁶ to account for the dimer axial ratio of 2:1. These values are in excellent agreement with the experimental τ_c of the "bound" water at pH 6.2 at 30°C and 2°C (Table 1).

Comparison of Results with Other Structural Information: Hydration and Dynamics of β -Lg Octamer. At pH 4.65, where the protein exists, to a large extent, as the octamer even at 30°C at concentrations above 0.01 g/ml, the Stokes radius of the "bound" water is about 30% less than the Stokes radius of the octamer itself. However, this value is still much closer to the theoretical value than those obtained by other investigators for other proteins.^{5,6,27-29} Furthermore, the 422-symmetry model for the octamer according to Timasheff and Townend²³ possesses a large central cavity which could accommodate trapped water; if the NMR experiment observed this trapped water, the τ_c value found would be less than that of the protein. Also, if the assumption is made that the NMR hydration of the octamer itself at 2°C equals $(\bar{\nu}_w)_{4.65} - (\bar{\nu}_w)_{6.2}$, values from 0.019 to 0.028 g H₂O/g protein can be calculated by the three methods described above. The total volume of the cavity, approximated by an internal sphere tangent to the subunits on the basis of known structural parameters,²³ amounts to about 6.5 nm³. Taking the specific volume of water as unity and thus its molecular volume as 0.03 nm³/molecule, this would correspond to about 220 moles H₂O/mole of octamer, or 0.027 g H₂O/g protein, which is within range of the NMR-derived hydration values for the octamer.

Since the derived NMR correlation times are number-average values, the hypothesis that the increase in hydration accompanying octamer formation is largely due to trapped water may be tested by calculating a number-average correlation time from the relationship $(\bar{\nu}_w)_{4.65} \tau_c = (\bar{\nu}_w)_{6.2} (\tau_c)_0 + [(\bar{\nu}_w)_{4.65} - (\bar{\nu}_w)_{6.2}] (\tau_c)_{cc}$, where $(\tau_c)_0$ is the correlation time of the octamer at 2°C (i.e. 145 ns, see Table 1), $(\tau_c)_{cc}$ is the correlation time of the central cavity of volume 6.5 nm³ (i.e. 1.4 ns), and $(\bar{\nu}_w)_{6.2}$ and $(\bar{\nu}_w)_{4.65}$ are the NMR hydration

values at pH 6.2 and 4.65, respectively. Calculation of τ_c from ^2H NMR hydration values by Method I at 2°C gives 36 ns, in fair agreement with the ^2H NMR experimental value of 32.2 ± 4.6 ns at pH 4.65 and 2°C . Although the results of this calculation furnish an indication of the reasonableness of the approach, they do not, however, show any exact mechanism of increased hydration accompanying octamer formation.

Anisotropic "Binding" Mechanism

The preceding calculations assume an isotropic relaxation mechanism, as detailed above. In the presence of salt, all the relaxation at pH 6.2 can be accounted for by a slow-tumbling and a fast-tumbling component, amounting to 13 and 204 moles $\text{H}_2\text{O}/\text{mole}$ dimer, respectively, and increasing at pH 4.65 and 2°C to 61 and 730 moles $\text{H}_2\text{O}/\text{mole}$ dimer; these may be considered reasonable values for the hydration of a protein.¹ This does not, however, eliminate the possibility of an anisotropic relaxation mechanism for "hydrodynamically bound" water. The present results may be interpreted equally well on the basis of the three-component derivation in conjunction with either a two- or three-"state" model and an appropriate order parameter, $S < 1$. Here a three-"state" model is defined, as for the isotropic mechanism, as comprising free-motion water, a slow-motion component (i.e. $\tau_c > 5$ ns), and a fast-motion component (i.e. $\tau_c = 48$ ps). For the latter, under extreme-narrowing conditions, the factor S^2 attached to Eq. [7] is changed to $(1 - S^2)$.⁷ The slow motion, in either the two or three-"state" anisotropic mechanism, may be due to such processes as protein reorientation, internal motion of the protein, or translational diffusion of water along the protein surface.^{7,30}

Reported values of $S = 0.06$ from ^{17}O relaxation⁷ have been derived by applying to a protein the line-splitting data obtained for a liquid crystal, on the assumption that 3 to 6 water molecules are "bound" to carboxyl groups and 1 to 3 to hydroxyl groups. Theoretical results of Walmsley and Shporer³⁰ give relationships for S (termed the scaling factor by these authors) based on ^1H , ^2H , and ^{17}O relaxation. From these, it follows that a value of $S = 0.06$ for ^{17}O would imply $S = 0.12$ for ^2H . At pH 6.2 (30°C and 2°C) and pH 4.65 (30°C and 2°C), one obtains hydrations, all in units of $\text{g H}_2\text{O}/\text{g protein}$, of 0.483, 0.500, 0.660, and 2.090 for the two-"state", and 0.295, 0.509, and 1.250 for the three-"state" model, respectively. A more reasonable estimate is obtained from the theoretical relationships of Walmsley and Shporer, together with the experimental results of Koenig et al.,^{5,6,27} which give $S = 0.23$ and corresponding hydrations, in the same units, of 0.119, 0.136, 0.180, 0.569, and 0.102, 0.105, 0.163, 0.435. The ΔH of hydration at pH 4.65 is found to be -6.8 for the two-"state" and -5.9 kcal for the three-"state" model. These results agree with the isotropic mechanism (Table 2), since S enters simply as a factor in the Kubo-Tomita-Solomon equations.

INFLUENCE OF PROTEIN HYDROPHOBIC SELF-ASSOCIATION AND ELECTROSTATICS: CASEINS, SUBMICELLES AND MICELLES

The major milk proteins, the caseins, occur in milk as colloidal particles. As viewed by electron microscopy, these particles are roughly spherical and have average diameters of 1200 to 1500 Å.^{31,32} One fundamental definition of a colloid is a group of particles in suspension, which interact with and are stabilized by their dispersion medium.³¹ Thus, the stability of the colloidal casein system must depend to a large extent on interactions with the major component of the dispersion system, water. This section will focus on the study of water-casein interactions in dynamic systems by nuclear magnetic resonance (NMR) relaxation techniques.

Experimental Procedures

Sample Preparation: Casein micelles were isolated from 2 l of fresh

warm milk to which 1 g of phenylmethylsulfonyl fluoride had been added to retard proteolysis. The milk was centrifuged at 4000 x g for 10 min to remove the cream fraction. Four hundred ml of this skim milk was centrifuged for 1 h at 88,000 x g (37°C). The pellets were washed twice in D₂O containing 25 mM piperazine-N,N'-bis(2-ethanesulfonic acid) (PIPES) (pH 6.75), 20 mM CaCl₂, and 80 mM KCl. The final protein concentration was fixed at about 100 mg/ml (total volume of 5 ml). Subsequent dilutions were made with the same buffer. To produce submicelles, sodium caseinate prepared from the same skim milk was dialyzed and lyophilized at pH 7.2; the lyophilized protein was dissolved in D₂O, in the same PIPES-KCl buffer without CaCl₂, but with added dithiothreitol to promote self-association of κ -casein.³³ These procedures were designed to minimize the concentration of H₂O in the D₂O solutions and to eliminate any significant contribution from deuterium exchange to the relaxation rates. Casein concentrations were determined spectrophotometrically on samples diluted 1/50 to 1/100 in 0.1 N NaOH; an absorptivity of 0.850 ml. mg⁻¹.cm⁻¹ at 280 nm was used for whole casein.^{33,34}

Casein Interactions

Although the primary structures of the bovine caseins³⁴ are now known, the structure of the colloidal casein micelle is still not fully clarified.^{31,32} From the point of view of the currently available data, the following model can be proposed.³⁵ At pH 6.75 with no calcium present, studies of the individual caseins have shown that they undergo hydrophobically driven self-associations which increase with increasing temperature and ionic strength. Studies on whole casein are limited but show similar results.^{32,33} Fig. 4 shows a proposed structure of three of these limiting polymers, which have overall radii of 90 Å and are commonly referred to as the submicellar form of casein.³⁵ Here, the hydrophobic core is considered to be composed mostly of the hydrophobic portions of α_s - and β -caseins, while κ -casein is considered to reside mostly at the surface, because of its ability to keep α_s - and β -caseins from precipitating at 37°C in the presence of calcium. All charged groups, including the serine phosphates, are located at or near the surface of the submicellar structure. In this model, the κ -casein content of the submicelles is variable. This representation (Fig. 4) is based upon small-angle X-ray scattering (SAXS) data³⁵ that indicate the presence of two nearly concentric regions of electron density for submicelles. Both centers exhibit relatively low protein electron density (high solvent occupancy). The inner, somewhat more electron dense area is dominated by hydrophobic interactions, while the outer, less dense area contains most of the charge and is highly hydrophilic.

Upon addition of calcium, the submicellar spherical particles are thought to self-associate through calcium phosphate salt bridges, which results in formation of a large colloidal spherical particle of approximately 650 Å radius, the micellar form of casein.^{31,32} The characteristic of κ -casein to be predominantly on the surface of the micelle has been shown by electron microscopy coupled with gold-labelled κ -casein,³¹ and with ferritin conjugate and anti- κ -casein.³⁶ While there is general agreement that micelles are formed from submicelles in the lactating mammary gland,³¹ a controversy exists as to whether or not the integrity of the submicelle is preserved upon the addition of calcium and secretion of milk. Moreover, physical chemical data suggest that in micelle formation, water is trapped within the micellar structure;^{1,37} the exact nature of this water is, however, uncertain.

Data from SAXS suggest³⁵ that the submicelles do retain a certain amount of their integrity, in that their scattering centers are relatively unchanged upon incorporation into micelles. The three submicelles shown in Fig. 4 represent the nearest-neighbor approach for the submicelles within the micelle. The micelle itself is then a repeat of this average substructure.³⁵ The degree of hydration predicted by SAXS argues for an extremely loose or hydrated overall structure. The water associated with this structure is most likely

Table 3. Hydration and Dynamics of Casein-"Bound" Water

	Temperature °C	τ_c ns	\bar{v}_w g H ₂ O/g proteins	R_{1b} s ⁻¹	R_{2b} s ⁻¹
Submicelle	30	38.9	0.00652	1904	10,510
	15	34.7	0.00824	2080	9,840
	2	29.8	0.01201	2323	9,070
Micelle	30	63.6	0.0165	1249	14,790
	15	51.1	0.0225	1515	12,570
	2	45.1	0.0282	1689	11,530

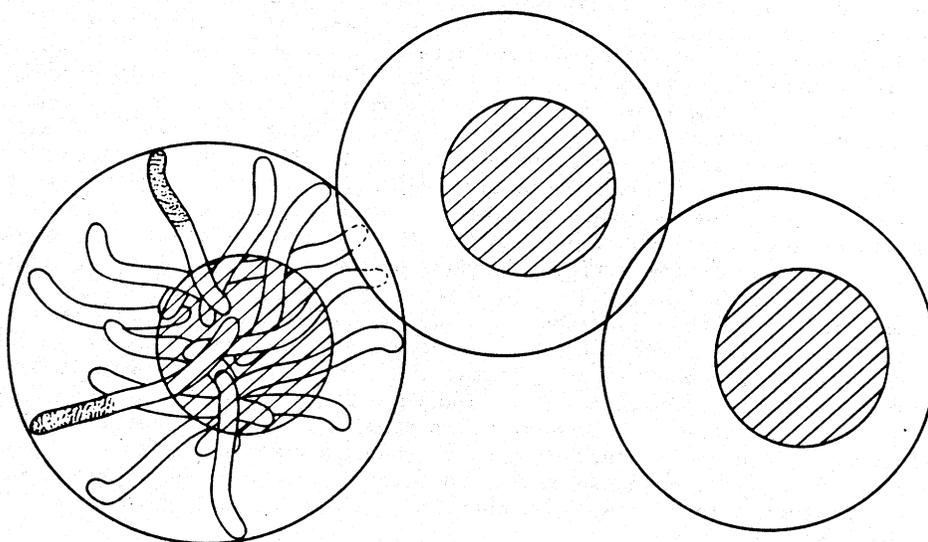


Fig. 4. Schematic representation of three submicelles within casein micellar structure. Cross-hatched area represents approximate core region of higher electron density and higher concentration of hydrophobic side chains. In lower-left particle, a few representative monomer chains are indicated. Shaded areas represent macropeptide portions of κ -casein.

not "tightly bound" but trapped by the condensation of the submicelles.

Given the success experienced in detecting trapped water in β -lactoglobulin by ²H NMR relaxation measurements,^{8,9} both longitudinal and transverse relaxation measurements of D₂O with varying concentrations of casein were performed, with and without calcium, at 30, 15, and 2°C. Fig. 5 shows R_1 and R_2 measurements at 15°C under submicellar and micellar conditions. All data were fitted by Equations [3] and [4] using a Gauss-Newton nonlinear regression program, as above. The experimental data and the data calculated from the model are in excellent agreement, as shown by the solid line in Fig. 5. Under these and all other conditions, the nonlinear portion of the curves yielded a virial coefficient of 0.0032 ± 0.0003 ml/mg, indicating the consistency of the experimental results. The linear portions of the curves were evaluated with a propagated standard error of about 4%; they contain the product of the relaxation rate of the "bound" water, the hydration, and fi-

nally the asymmetry parameter, S . These will be separated, and each will be discussed in the following sections.

Hydration and Dynamics of Casein: Isotropic Model

From the linear portion of the longitudinal and transverse relaxation results, Equation [3], and the Kubo-Tomita-Solomon equations, Equations [5] and [6], the following parameters for the caseins were calculated at the various environmental conditions:

- 1 - correlation times, τ_c ; while these are primarily NMR parameters arising from Equations [5] and [6], in previous work,^{8,9} τ_c has been shown to be related to the rotational speed of water "bound" to the protein;
- 2 - hydration values, $\bar{\nu}_w$; expressed in g H₂O "bound" per g of protein, for $S = 1$ in Equation [7]. This latter value implies that water is held on the surface and is thus not moving independently of the motion of the protein (Isotropic Model);
- 3 - relaxation rates, R_{1b} and R_{2b} ; the rates for water "bound" to the protein, as defined above in 1 and 2.

The results of the analyses for these parameters are shown in Table 3. Here, $\bar{\nu}_w$ values increased from 0.00652 to 0.01201 g water/g protein, and τ_c values decreased from 38.9 to 29.8 ns, as the temperature decreased from 30 to 2°C for casein in the submicellar form; propagated standard errors were about 8% for τ_c and 6% for $\bar{\nu}_w$. The same temperature dependence of τ_c and $\bar{\nu}_w$ was exhibited under micellar conditions, although at all temperatures, their absolute values were larger for the micellar form than for the submicellar form.

At this point, it would be appropriate to note that although the caseins are self-associating, we need to consider here only the aggregated form. The concentrations used were high enough so that the association equilibrium favors polymer formation.³³ For both micelles and submicelles, no significant differences in hydration would result from protein concentration-dependent dissociation effects at 30°C. (At lower temperatures, this must be qualified, as discussed below.) Also, these $\bar{\nu}_w$ values will, in all probability, show only a fraction of the total hydration, since at 9.17 MHz, any "bound" water with $\tau_c < 6$ ns would have a R_2/R_1 ratio of unity and would not be observable by this methodology.

Since it has been shown previously that the τ_c values derived from NMR relaxation results are those for the unhydrated rather than the hydrated form of the protein,^{8,9} the Stokes radius, r , calculated from τ_c values using the Stokes-Einstein relationship, would indeed be a representation of the quaternary structure for the unhydrated protein. Such r values were calculated from all τ_c results and are listed in Table 4 for the caseins.

A Stokes radius of 36.4 Å (Table 4), found at 30°C, is at the lower limit of radii reported for submicelles, whose sizes range from 40 to 90 Å, depending on the method of measurement.^{33,38,39} (It should be noted that direct comparison between Stokes radii derived by this NMR method and those calculated from small-angle scattering (SAXS) data is somewhat inappropriate, because these latter radii include all water of hydration, whereas the NMR values^{8,9} pertain to the anhydrous protein. Nevertheless, the Stokes radius calculated from SAXS for the inner core depicted in Fig. 4 is 38 Å.³⁵ It could be that "tightly bound" water exists near the surface of the inner core.) Variations in this hydration parameter are of interest, when viewed in light of changing structures. Results for the submicelles show a decreasing hydration value (Table 3) and an increase in the Stokes radius (Table 4) with increasing temperature. This suggests that hydrophobic interactions are involved in the formation of the submicelles, since as the temperature is raised, water is excluded from the hydrophobic interface during an association process.

Table 4. Molecular Parameters of Caseins Derived from Data of Table 3

	Temperature °C	r Å	M _P	$(\bar{\nu}_w)_r$	S	$(\bar{\nu}_w)_s = 0.237$
Submicelle	30	36.4	165,000			0.116
	15	30.5	97,200			0.147
	2	25.5	56,800			0.214
Micelle	30	42.9	270,700	0.469	0.188	0.294
	15	34.8	144,500	0.357	0.251	0.400
	2	29.3	86,200	0.380	0.272	0.502

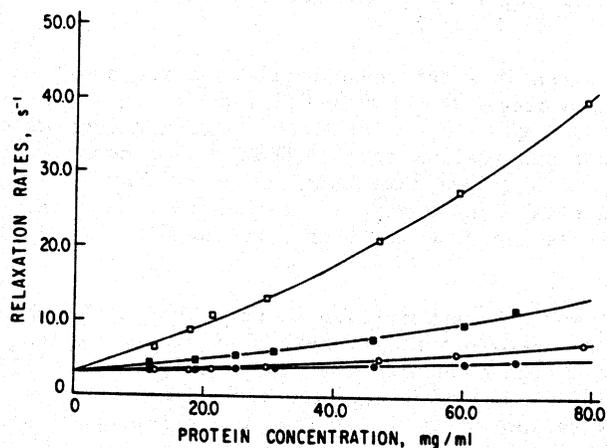


Fig. 5. Dependence of deuteron relaxation rates of water on casein concentration in D₂O at pH 6.75 in PIPES-KCl buffer at 15°C: -●-●-, R₁ measurements, submicellar form; -■-■-, R₂ measurements, submicellar form; -○-○-, R₁ measurements, micellar form; -□-□-, R₂ measurements, micellar form.

Although the absolute value of the Stokes radius, calculated from τ_c , for the micelle is of the same order of magnitude as that of the submicelle, it was not as large as would be expected, because of instrumental limitations. These limitations are due to the large size of the casein micelle ($r = 650 \text{ \AA}$), which would result in a τ_c value of nearly $200 \mu\text{s}$. Such a slow motion would yield a transverse relaxation rate too large to be seen by NMR experiments at 9.17 MHz. In one sense, what the data may show is the average hydration of the caseins within the micelle, since the fastest motions dominate relaxation data. The micelle exhibits the same temperature dependence as the submicelle, suggesting hydrophobic interactions, in agreement also with previous investigators who theorized that micelles are formed by aggregation, via Ca^{2+} salt bridges, of submicelles.^{31,32} The slight increase in r from submicelle to micelle is probably due to a gradual increase in internal hydration (trapped water) as the submicelle is incorporated into the micelle. This is also in agreement with the extraordinary hydration (2 to 7 g H₂O/g protein) found by SAXS³⁵ and by hydrodynamic measurements^{37,40} for the casein micelle.

As noted above, caseins, both in micellar and submicellar forms, show

decreased hydration on going from 2°C to 30°C. This correlates well with changes in the viscosity of whole skim milk.⁴¹ The relative viscosity of skim milk decreases dramatically over this temperature range (2°-30°C); this change has been correlated⁴¹ with lowered hydration due to a decrease in the volume occupied by the micelles (their voluminosity). The NMR hydration (Table 3) parallels these changes. At first sight, the increase in NMR radius (Table 4) with temperature would seem contradictory; however, it is not the total hydrodynamic radius which is increasing, but the size of the hydrophobic core (the "anhydrous protein radius"), as the hydrophobically driven associations increase at from 2° to 30°C. In a sense, the caseinate are becoming more compact as the temperature increases.

Derived Molecular Parameters of the Caseins: Since it has been shown that the Stokes radius of "bound" water derived from NMR relaxation results can be related to the anhydrous volume,^{8,9} a molecular weight of the caseins can be calculated from

$$M_p = 4/3 \pi r^3 N / \bar{v}_p,$$

where r is the Stokes radius (Table 4), N is Avogadro's number, and \bar{v}_p is the average partial specific volume of the caseins,³⁵ taken here to be 0.736. The results are presented in Table 4. Here, the increase in M_p for both the submicelle and the micelle, as the temperature is increased, is a qualitative indication of hydrophobic self-association, not only for the submicelle but also within the micelle structure itself.

To quantitate this temperature-dependent variation of M_p , apparent equilibrium constants, K_A , were calculated from $K_A = M_p/23,300$, where 23,300 is the average monomer molecular weight of casein. (This relationship is reasonable, since the measurements were performed at high concentrations of casein, where the equilibrium is driven nearly completely toward the aggregated form.) K_A and the temperature, T , were then used in the van't Hoff expression, according to which the slope of a plot of $\ln K_A$ vs $1/T$ yields the apparent enthalpy of self-association, ΔH , for submicellar and micellar casein (see Fig. 6). The van't Hoff plots for the two forms of casein are essentially parallel. In fact, ΔH values for the submicelle formation were calculated to be 6.34 ± 0.11 kcal, and only slightly higher, 6.81 ± 0.28 kcal, for the self-association to the micelle.

These values are in good agreement with ΔH of 4.67 found for the association of purified α_{s1} - and κ -caseins.⁴² This quantitation of the temperature variation of the self-association strongly suggests that the integrity of the submicelle is at least partly preserved when it is incorporated into the micellar form by calcium-phosphate salt bridges.

Hydration: Anisotropic Tumbling Model

Up to this point, we have calculated all NMR hydration values using an isotropic motion mechanism for the "bound" water. However, the motion of the "bound" water may, in fact, be anisotropic (i.e. not identical to that of the protein with $S < 1$ in Equation [7]). This will be true if the correlation times are long with respect to the Larmor frequency used in these experiments. Such may be the case for the casein micelles, where water may be trapped at the surfaces of submicelles as they self-associate via calcium-phosphate salt bridges into micelles. An attempt has been made to estimate the asymmetry factor, S , for casein using the following rationale. If it is assumed that the increased radii of the micelles, relative to the submicelles at the same temperature (Table 4), are due to increased hydration caused by micelle assembly, then using the partial specific volume of the caseins, new hydration values for the micelle due to assembly can be calculated;³⁷ these values are given in Table 4, column 4, $(\bar{v}_w)_r$. From these latter values and the isotropic hydration values of Table 3, column 3, S values were calculated

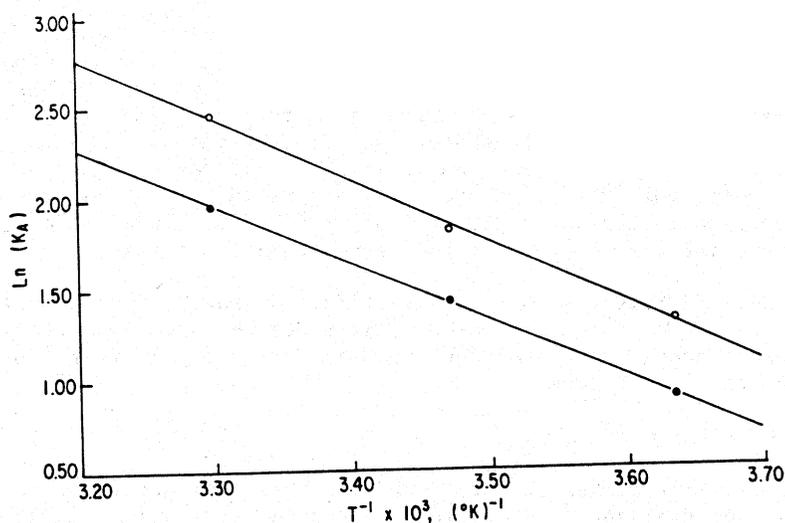


Fig. 6. Van't Hoff plots for temperature dependence of the self-association of casein: -●-●-, submicellar form; -○-○-, micellar form.

at 30°, 15°, and 2°C. The S values for casein micelles are listed in Table 4 and average 0.237 ± 0.033 , in good agreement with the value of 0.23 predicted for anisotropic motion by Walmsley and Shporer.³⁰ Therefore, new hydration values, $(\bar{\nu}_w)_s = 0.237$, can be calculated for an anisotropic motion mechanism using the $\bar{\nu}_w$ values of Table 3 and the average S of 0.237. These are listed in the last column of Table 4. The absolute values of these new hydrations, ranging from 0.116 to 0.502 g water/g protein for submicellar and micellar casein, are closer to the expected hydration values derived by other methods,^{9,40} and follow known changes in micelle voluminosity,⁴¹ described above.

It should be stressed that, although the above calculation is not proof of the existence of water with anisotropic motion "bound" to casein, it does furnish significant information. It would not be unexpected that, in a large porous structure such as the casein micelle, water could be "bound" to the protein components but show some partial motion of its own. What is important here is the variation of hydration with quaternary structural changes of the casein, rather than absolute values of hydration. It may be that the absolute value of the hydration derived from NMR relaxation results will be obtained only at some future time, when the controversy regarding the isotropic vs. anisotropic nature of water "binding" to proteins in solution is resolved. At any rate, water "bound" at the surface of the micelles or influenced by the slow motion of the large micellar particle would not be sensed at the frequencies used here.

CONCLUDING REMARKS

The foregoing considerations add up to considerable agreement between certain theoretically and experimentally derived quantities. However, none of the above arguments should be interpreted as proof of any particular NMR mechanism or model, nor of the identity of the particular groups on the protein surface that interact with water. Even without such conclusions, and in place of the quest for absolute values of hydration, it can be useful to scrutinize relative changes in hydration, when these can be taken as functions of changes in secondary, tertiary, or quaternary structure of a protein.

However, it must be stressed that the use of a frequency-dependent NMR spectrometer in lieu of protein concentration-dependent relaxation measurements at a minimum of two frequencies can yield erroneous results. High virial effects can indeed be present in protein solutions, especially if salt is not added to the system in order to minimize the protein-protein electrostatic potential. However, by use of nonlinear regression analysis, the virial coefficient of the protein, which is related to its net charge, can also be extracted from these protein concentration-dependent relaxation data.

Furthermore, although the NMR relaxation mechanism controversy still exists between the isotropic binding model and the intermediate asymmetry model, it can be concluded from all the above results that the "hydrodynamically influenced water" model of Koenig et al.^{5,6,13} can finally be placed to rest. It is suggested here that only a model whereby water directly "binds" to proteins can account for the water relaxation rate experiencing the charge of the macromolecule in protein solutions and for the protein structural results presented in this chapter. In addition, the model of water moving from site to site on the asymmetric surface of a macromolecule with a fast-motion component equal to the diffusion coefficient of "unbound" water is also believed to be unlikely in protein solution. With the abundance of "free water" (i.e. 55.6 moles/liter of water) relative to the protein concentration (approximately 10^{-3} moles/liter), such a phenomenon would be thermodynamically unsound. In contrast, in experiments involving protein powders and other two-phase biological systems, where only a limited amount of water is added to a solid or amorphous material and where surface adsorption of water to an insoluble second phase can take place, the above mechanism of asymmetric motion along the macromolecular surface most likely does predominate.

Therefore, in a true protein solution, only the isotropic two-"state" binding mechanism or the water "binding" with a fast motional component to a macromolecule with a slow anisotropic motion are suggested to be the most reasonable models of choice at this time.

One point about the above results should be noted here. The use of the ^2H NMR experiments, which, of course, yield a sharp and well-defined water peak, can yield an extra relaxation resulting from fast-exchangeable protein protons, such as those arising from arginine, lysine, and even aspartic and glutamic acid side chains. However, in most cases, a calculation from the amino-acid sequence of the protein in question can show the percentage contribution of this term to the results, and in most cases, it will be small. Nevertheless, in the above studies, this effect would still not account for the change in hydration with temperature, as observed for casein self-association or for the β -lactoglobulin tetramerization. However, investigators may also perform experiments using ^{17}O NMR relaxation of the samples of protein dissolved in D_2O , if they are concerned with the magnitude of the exchange contribution to the hydration term.

Finally, the above absolute hydration results, using a two-"state" isotropic "binding" model, are much lower than hydration values determined by small-angle X-ray scattering and sedimentation velocity experiments. It appears that only an asymmetric "binding" model could bring the order of magnitude of these hydration results into line with SAXS and hydrodynamic values. However, SAXS and hydrodynamic results may also contain another contribution not considered by some investigators. In fact, there may also be a dynamic contribution to the hydration, since it is measured by the difference between two volumes. The concept of protein 'breathing' has been emphasized before,⁴³ and the entire topic of protein dynamics has been reviewed recently.^{3,44} The effects of dynamic changes such as fluctuations (e.g. ring flipping and domain hinge bending) on packing volumes and accessible surface areas remain unclear. Progress on these questions may come from dynamic modeling by computer simulation.

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