

Water Interactions with Bovine Caseins by Hydrogen-2 Nuclear Magnetic Resonance Relaxation Studies: Structural Implications

HAROLD M. FARRELL, JR., HELMUT PESSEN,
and THOMAS F. KUMOSINSKI
US Department of Agriculture
Agricultural Research Service
Eastern Regional Research Center
Philadelphia, PA 19118

ABSTRACT

A method was developed for determining hydration from the protein concentration dependence of deuterium magnetic resonance relaxation rates. Measurements were made in D_2O on both casein micelles and submicelles. 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, hydration and correlation times were calculated for the proteins; from the latter parameter the Stokes radius was obtained. Molecular weights, calculated from the Stokes radius using the Stokes-Einstein relationship and the partial specific volume, were in the range of those published for caseins in the absence of Ca^{2+} (submicelles). For casein submicelles variations of Stokes radius and hydration with temperature were in agreement with hydrophobically driven aggregations and in accord with known changes in molecular state. Similar temperature variations of Stokes radius and hydration were observed for casein micelles; however, their absolute values, although greater than those of submicelles, were less than expected. These data are interpreted with respect to a model in which trapped water in the micelles is associated with discrete submicellar structures.

INTRODUCTION

Water is the universal solvent for biological fluids, yet its basic structure in these systems

remains an area of controversy. Even in a well-studied system such as milk, where the water content (actually the freezing point) is one legal definition of the commodity, the interactions of water with proteins and other components are poorly understood. The major milk proteins, the caseins, occur in milk as colloidal particles. As viewed by electron microscopy (Figure 1), these particles are roughly spherical and have average diameters of 1200 to 1500 Å (10, 26). One fundamental definition of a colloid is a group of particles, in suspension, that interact with and are stabilized by their dispersion medium (21). 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.

Although electron micrographs such as the one shown in Figure 1 portray the micelles as spheres, we must not forget that these micelles are porous structures in dynamic equilibrium with their dispersion medium, the milk serum (10, 21). This paper will focus on one method for studying water in dynamic systems, nuclear magnetic resonance (NMR) relaxation techniques. These methods can be used to gain structural information and also address more practical considerations, such as the role of protein in suppressing water activity.

MATERIALS AND METHODS

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 $4000 \times g$ for 10 min to remove the cream fraction. Four hundred milliliters of this skim milk was centrifuged for 1 h at $88,000 \times g$ ($37^\circ C$). The pellets were washed twice in D_2O containing 25 mM piperazine-N,N'-bis(2-ethanesulfonic acid)

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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- μ l sample bulb, available from Wilmad Glass Co., Inc., Buena, NJ. 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 that 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 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 FX60Q 10-mm $^1\text{H}/^{13}\text{C}$ dual probe insert.

Longitudinal relaxation rates R_1 were measured by the inversion-recovery method (34), where the repetition time T in the pulse sequence $[\dots T \dots \pi \dots \tau \dots \pi/2 \dots]$ was chosen to be at least five times T_1 ($\equiv R_1^{-1}$) and the values of the variable delay time τ ranged from 10 ms to 3 s 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:

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

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 (Figure 2, 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 to 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 measurement (9) of $R_{1\rho}$, the longitudinal relaxation rate in the rotating frame. $R_{1\rho}$ 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

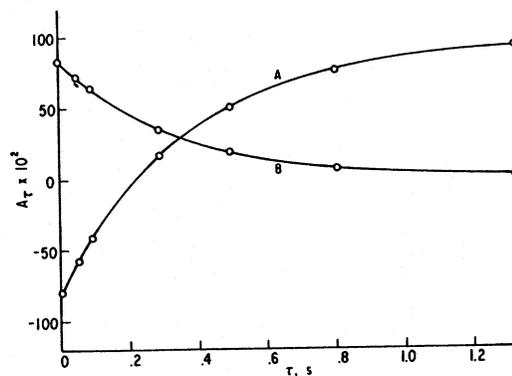


Figure 2. Deuteron resonance peak intensities, A_τ , as a function of time, τ , for casein micelle solution at pH 6.75, 15°C, in D_2O (78.5 mg/ml) and in piperazine- $\text{N,N}'$ -bis(2-ethanesulfonic acid)- KCl - CaCl_2 buffer. A. Longitudinal relaxation measurements (R_1) from inversion-recovery method. 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 (Equations [1] and [2]) to experimental intensities by non-linear regression.

radio-frequency field in the rotating frame (9); this was the case, within the limits of experimental error, in the present work. The R_2 was evaluated as described for R_1 , except that the relation between peak intensity A_T and decay time τ in this case becomes:

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

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 (Figure 2, curve B).

For each sample, R_2 was determined with the same number of replications as R_1 ; standard errors amounted to 2 to 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 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 D₂O to eliminate cross relaxation effects between water and protein protons, such as observed by Edzes and Samulski (7), and by Koenig et al. (14). Residual H₂O in the sample will have little effect on the D₂O rates since even at elevated (1:1) ratios, D₂O relaxation is not compromised by the presence of H₂O (14). However, in samples prepared as described herein, the residual H₂O is always less than 5%.

Analysis

For a two-state model (bound and free water), Kumosinski and Pessen (16) 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 ,

$$R_{Obs} - R_f = (R_b - R_f) \bar{\nu}_w a_p / W \quad [3]$$

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. The $\bar{\nu}_w$ 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 grams per gram. Also:

$$a_p = c \exp(2B_0 c + \dots) \quad [4]$$

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 (15, 28),

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

and

$$R_{2b} = K\tau_c [3 + 5(1 + \omega_0^2 \tau_c^2)^{-1} + 2(1 + 4\omega_0^2 \tau_c^2)^{-1}] \quad [6]$$

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.,

$$K = (3/80)(e^2 qQ/\hbar)^2 (\eta^2/3 + 1)^{-1} S^2 \quad [7]$$

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 (1), and S is the order parameter for intermediate asymmetry of the motion of the bound water (12). 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 $\eta = 0$, $\nu_0 = 9.17$ MHz, and $e^2 qQ/\hbar = 215.6$ kHz (35).

RESULTS AND DISCUSSION

Water and Nuclear Magnetic Resonance Relaxation

The individual molecules of water, although constrained by water structure, as discussed in the previous paper by Meyers (20), 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 (Figure 3) can rotate about its own axis once every 3 ps; in addition, it can move rapidly through the water matrix, as shown by Avbelj et al. (2), on a scale of femtoseconds. If we impose a properly selected external magnetic field on the water, 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 (18). 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 Materials and Methods, 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) increase as a function of added protein concentration. This is shown in Figure 4 for sodium caseinate (submicelles) and, more dramatically, for

CORRELATION TIMES OF FREE AND BOUND WATER

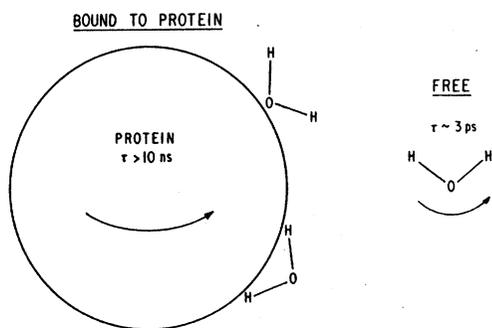


Figure 3. Schematic representation of a water molecule and a protein molecule with water bound to it. The diagram also indicates the approximate rotational correlation times (τ) of free water and protein-bound water.

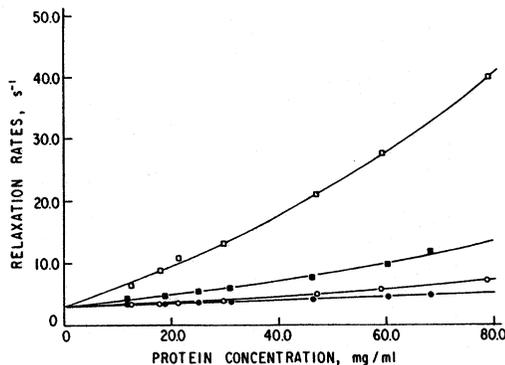


Figure 4. Dependence of deuteron relaxation rates of water on casein concentration in D_2O at pH 6.75 in piperazine- N,N' -bis(2-ethanesulfonic acid)-KCl buffer at 15°C. R_1 measurements, submicellar form (\bullet); R_2 measurements, submicellar form (\blacksquare); R_1 measurements, micellar form (\circ); R_2 measurements, micellar form (\square).

casein micelles. This is as predicted from Equation [3]. The reasons for this phenomenon are quite complex, and the theory and testing of the hypotheses connected to this have been demonstrated (16) and reviewed in detail elsewhere (24). Briefly, a simple explanation is as follows:

1. Water, bound to the protein, has a different rate of relaxation than free water (Figure 3); the bound water moves essentially with the more slowly rotating protein (e.g., nanoseconds for β -lactoglobulin).
2. Free water and bound water rapidly exchange so that excited free water can bind to the protein, give up its energy (relax), and return to the solution in femtoseconds and vice versa.

Other mechanisms of relaxation can occur and can obscure whether or not NMR-relaxation measurements can measure bound water. Perhaps the most serious consideration of these is the phenomenon of cross relaxation (7, 14, 24, 25). Because 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 caseins. 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 (24). For this work we will concentrate only on a simple two-state model.

β -Lactoglobulin-Water Interactions

Much earlier work from this laboratory on water-protein interactions (16, 25) centered on the whey protein β -lactoglobulin, primarily because the wealth of structural information available concerning this protein made interpretation of the NMR relaxation data easier.

β -Lactoglobulin has been reported to occur naturally in five genetic variants (8). The A and B forms used in our studies undergo a variety of changes in conformation and state of association (summarized in Figure 5). The present discussion focuses on one of these: the rapid dimer \rightleftharpoons octamer equilibrium (29, 30, 31, 32), occurring primarily in the cold. The two subunit, 36,700-dalton dimer is the kinetic unit persisting over a wide range of moderate conditions of pH from 3 to about 7.

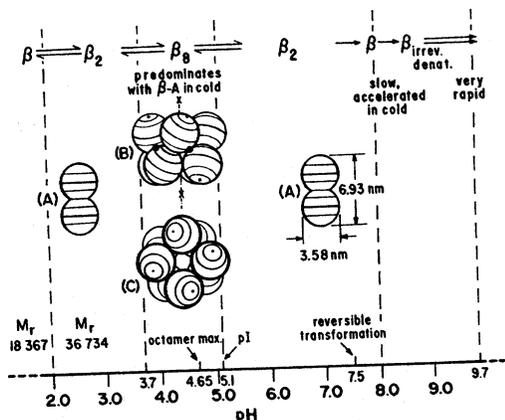


Figure 5. Schematic representation of changes in the structure of β -lactoglobulins as a function of pH. β = β -Lactoglobulin. Insets (molecular models): (A), dimer; (B), octamer, with square decahedral faces on top and bottom; octamer bonds (\bullet); tetrad axis ($x-x$); circular lines indicate monomer equators and parallels perpendicular to the dimer axes; (C), octamer, with square faces in front and back, tetrad axis perpendicular to plane of paper. [Based on data of (11, 29, 30, 31, 32)].

Between pH 3.7 and 5.1, self-association of β -lactoglobulin dimer to octamer occurs as the temperature decreases. This is a rapid equilibrium process that is well-characterized (2, 29, 30, 31, 32); it occurs to a greater extent with the A variant than with the others. Because self-association involves protein-protein interactions, which should remove some of the potential water-binding sites on the dimer surface, a decrease in hydration might be expected on formation of the octamer. In contrast, the hydration measured by ^2H NMR relaxation (16) actually increases (Table 1). The observed increased hydration requires an examination of the molecular geometry. The octamer, as determined by Timasheff and Townend (30), is a closed ring, consisting of four dimers associated symmetrically about a tetrad axis; its general shape is that of a decahedron with a substantial central cavity (Figure 5). Because of a sizable hole (approx. 1.4 nm diameter) in each of the two square faces of the decahedron (Figure 5, Inset C), the water in the interior of the octamer is readily accessible for fast exchange with the bulk water on the outside, as we assumed (see Proteins and Water Relaxation).

TABLE 1. Hydration of β -lactoglobulin A as calculated from deuterium nuclear magnetic resonance.¹

pH	Temperature	$\bar{\nu}_w$ (g H ₂ O/g protein)
	(°C)	
6.2	30	.0063
	2	.0072
4.65	30	.0095
	2	.0301

¹ $\bar{\nu}_w$ = degree of hydration.

An estimate of the amount of water contained in the cavity may be obtained from geometric parameters (30), which indicate that an inscribed sphere, tangent to the interior van der Waals surfaces of the octamer, would have a volume of about 6.45 nm³. (This estimate neglects the spaces in corners and crevices of the cavity; these will be largely offset by the dimer-dimer contact areas between the eight monomer units made unavailable to water binding upon tetramerization at the 12 new protein-protein contact sites.) From the molecular weight and volume of water and the molecular weight of the protein one finds that this cavity corresponds to .0264 g H₂O/g protein. It can, therefore, accommodate a considerable amount of water detectable by NMR, giving rise to the increased hydration observed under the conditions where octamer formation occurs. There is thus good agreement between the calculated hydration and that measured by NMR shown in Table 1 for the octamer at pH 4.65 and 2°C.

Casein Interactions

Although the primary structures of the bovine caseins (8) are now known, the structure of the colloidal casein micelle is still not fully clarified (10, 26). From the currently available data the following model can be proposed (26). At pH 6.75 with no calcium present, individual caseins undergo hydrophobically driven self-associations, which increase with increasing temperature and ionic strength (26). Studies on whole casein are limited but show similar results (23, 26). Figure 6A shows one proposed structure of this limiting polymer, commonly referred to as the submicellar form of casein.

Here, the hydrophobic core is considered to be composed mostly of the hydrophobic portions of α_s - and β -caseins, whereas 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 on the surface of the submicellar structure. In this model the κ -casein content of the submicelles is variable.

Upon addition of calcium, these submicellar spherical particles are thought to self-associate owing to calcium phosphate salt bridges, which result in formation of a large colloidal spherical particle of approximate radius of 650 Å, the micellar form of casein (see Figure 6B). A controversy exists as to whether or not the integrity of the submicelle is preserved upon the addition of calcium. Although it is widely accepted that trapped water exists within the micellar structure (19, 37), the nature of this water is uncertain. 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 (26) and with ferritin conjugate and anti- κ -casein (4).

Given the success experienced in detecting trapped water in the β -lactoglobulin work (16, 25), [²H] NMR relaxation measurements, both longitudinal and transverse, of D₂O with varying concentrations of casein, were performed with and without calcium at 30, 15, and 2°C. Figure 4, as noted, shows R₁ and R₂ 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 developed at this laboratory. The experimental data and the data calculated from the model employed are in excellent agreement, as shown by the solid line in Figure 4. Under these and all other conditions the nonlinear portion of the curves yielded a virial coefficient of .0032 ± .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 finally the asymmetry parameter, S. These will be separated and each will be discussed in the following sections.

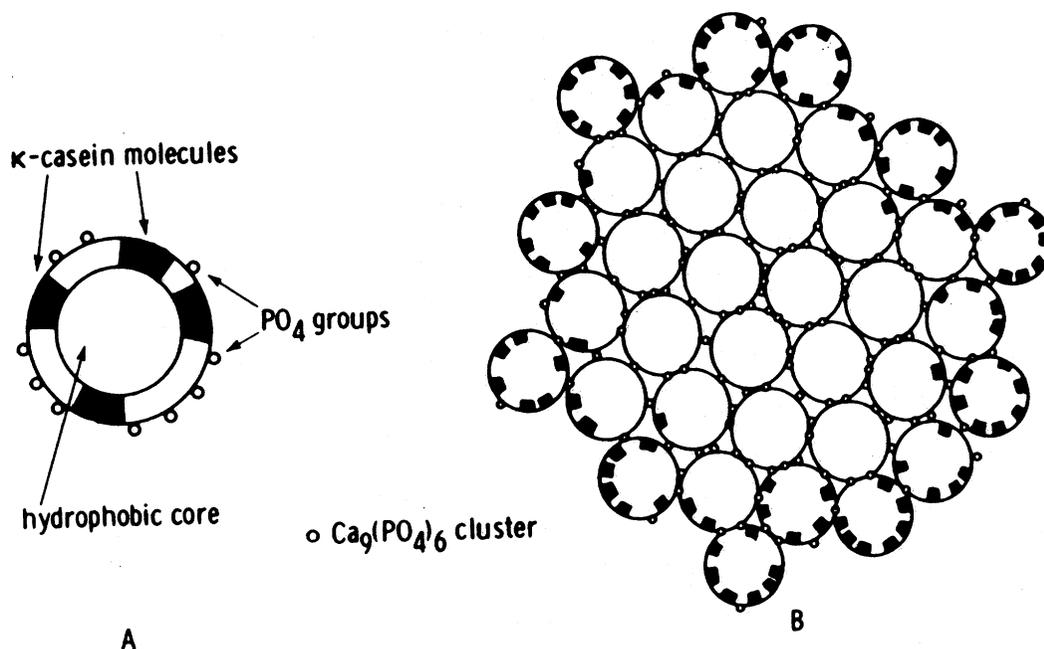


Figure 6. Quaternary structural forms of casein. A, submicellar form; B, micellar form upon addition of calcium. [(From (26), by permission)].

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 were calculated at the various environmental conditions, for the caseins:

1. Correlation times, τ_c . Although these are primarily NMR parameters arising from Equations [5] and [6], in previous work (16), τ_c has been shown to be related to the rotational speed of water bound to the protein (Figure 3);
2. Hydration values, \bar{v}_w , expressed in grams H₂O bound per gram 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} , are the rates for water bound to the protein as defined in items 1 and 2.

The results of the analyses for these parameters

are shown in Table 2. Here, \bar{v}_w values increased from .00652 to .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{v}_w . The same temperature dependence of τ_c and \bar{v}_w was exhibited under micellar conditions, although at all temperatures their absolute values were larger for the micellar form than for the submicellar form.

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 (23). 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{v}_w 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.

TABLE 2. Hydration and dynamics of casein bound water.¹

	Temperature	τ_c	$\bar{\nu}_w$	R_{1b}	R_{2b}
	(°C)	(ns)	g H ₂ O/g protein	(s ⁻¹)	
Submicelle	30	38.9	.00652	1904	10,510
	15	34.7	.00824	2080	9840
	2	29.8	.0120	2323	9070
Micelle	30	63.6	.0165	1249	14,790
	15	51.1	.0225	1515	12,570
	2	45.1	.0282	1689	11,530

¹ τ_c = Correlation time of bound water, $\bar{\nu}_w$ = degree of hydration, R_{1b} = longitudinal, and R_{2b} = transverse relaxation rates of bound water.

Because the τ_c values derived from NMR relaxation results are those for the unhydrated rather than the hydrated form of the protein (16, 24), 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 3 for the caseins.

The r of 36.4 Å (Table 3) 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 (23, 26, 27). (Direct comparison between r derived by this NMR method and those calculated from hydrodynamic or small-angle scattering data would be inappropriate, because these latter include all water of hydration, whereas the NMR values (16, 24) pertain to the anhydrous protein. Nevertheless, changes in this parameter

accompanying protein structural changes are of interest in helping to establish structural information.) Results for the submicelles show a decreasing $\bar{\nu}_w$ (Table 2) and an increase in the r (Table 3) 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.

Although the absolute value of the r calculated for the micelle is on 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 ($r = 650$ Å), which would result in a τ_c of nearly 200 μ s. Such a slow motion would yield a transverse relaxation rate too large to be seen by these NMR experiments at 9.17 MHz. In one sense, what the data may show is the average

 TABLE 3. Molecular parameters of caseins derived from data of Table 2.¹

	Temperature	r	M_p	$(\bar{\nu}_w)_r$	S	$(\bar{\nu}_w)_s = .237$
	(°C)	(Å)				
Submicelle	30	36.4	165,000			.116
	15	30.5	97,200			.147
	2	25.5	56,800			.214
Micelle	30	42.9	270,700	.469	.188	.294
	15	34.8	144,500	.357	.251	.400
	2	29.3	86,200	.380	.272	.502

¹ r = Stokes' radius, M_p = molecular weight, $(\bar{\nu}_w)_r$ = new hydration due to assembly, S = symmetry factor for anisotropic motion, $(\bar{\nu}_w)_s$ = hydration for anisotropic motion mechanism.

hydration of the caseins within the micelle, since the fastest motions dominate relaxation data. The micelle exhibits the same temperature dependence as the submicelle, showing hydrophobic interactions, in agreement also with previous investigators who theorized that micelles are formed by aggregation, via Ca^{2+} salt bridges, of submicelles (10, 26). 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 $\text{H}_2\text{O}/\text{g}$ protein) found by hydrodynamic measurements (13, 37) for the casein micelle.

As noted, caseins in micellar and submicellar forms show decreased hydration on going from 2 to 30°C. This correlates well with changes in the viscosity of whole skim milk (6, 38). The relative viscosity of skim milk decreases dramatically over this temperature range (2° to 30°C); this change has been correlated (6, 38) with lowered hydration due to a decrease in the volume occupied by the micelles (their voluminosity). The NMR hydration (Table 2) parallels these changes. At first sight the increase in NMR radius (Table 3) with temperature would seem contradictory; however, it is not the total hydrodynamic radius that is increasing, but the size of the hydrophobic core – the “anhydrous protein radius” as the hydrophobically driven associations increase at 30°C. In a sense, the caseinates are becoming more compact as the temperature increases.

Derived Molecular Parameters of the Caseins

Because the r of the bound water derived from NMR relaxation results can be related to the anhydrous volume (16, 24), a molecular weight of the caseins can be calculated from:

$$M_p = 4/3 \pi r^3 N / \bar{v}_p \quad [8]$$

where r is the Stokes radius (Table 2), N is Avogadro's number, and \bar{v}_p is the average partial specific volume of the caseins, taken here to be .736 (18). The results are presented in Table 3. 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

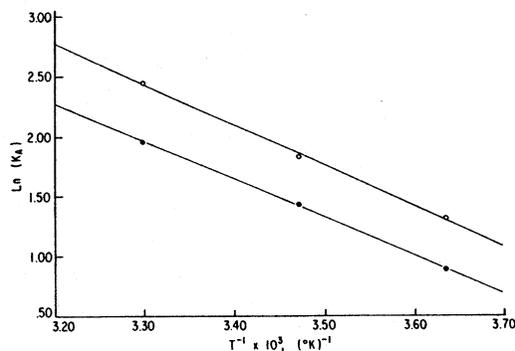


Figure 7. Van't Hoff plots for temperature dependence of the self-association of casein. Submicellar form (\bullet); micellar form (\circ).

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.) The $\ln K_A$ and the inverse temperature ($1/T$) were then used with the van't Hoff expression (see Figure 7) to calculate the apparent enthalpy of self-association, ΔH , for submicellar and micellar casein (18). As can be seen in Figure 7, 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 $6.34 \pm .11$ kcal and only slightly higher at $6.81 \pm .28$ kcal for the self-association in the micelle. These values are in good agreement with ΔH of 4.67 found for the association of purified α_{s1} - and κ -caseins (5). 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. Moreover, extrapolation of the van't Hoff plot to 37°C yields an apparent molecular weight for the submicelles of 210,000, which is in excellent agreement with results from other investigations (3, 22, 26).

Hydration: Anisotropic Tumbling Model

Up to this point we have calculated \bar{v} using an isotropic motion mechanism of 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 (18) using the following rationale. If it is assumed that the increased radii of the micelles relative to the submicelles at the same temperature (Table 3) are due to increased hydrations caused by micelle assembly, then using the partial specific volume of the caseins, new hydrations for the micelle due to assembly can be calculated (18); these values are given in Table 3 column 4 (\bar{v}_w)_r. From these latter values and the isotropic hydrations of Table 2 column 3, S values were calculated at 30, 15, and 2°C. The S values for casein micelles are listed in Table 3 and average $.237 \pm .033$, in good agreement with the .23 predicted for anisotropic motion by Walmsley and Shporer (36). Now, new hydrations (\bar{v}_w)_s = .237 can be calculated for an anisotropic motion mechanism using the \bar{v}_w values of Table 2 and the average S of .237. These are listed in the last column of Table 3. The absolute values of these new hydrations, ranging from .116 to .502 g water/g protein for submicellar and micellar casein, are closer to the expected hydration values derived by other methods (16, 24).

It should be stressed that although the calculation in the previous paragraph is not proof of the existence of water with anisotropic motion bound to casein, it does furnish significant information. In a large porous structure such as the casein micelle water could be expected to be bound to the protein components and also to 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 their absolute values. It may be that the absolute

value of the hydration derived from NMR relaxation results will be obtained only 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 particle would not be sensed at the frequencies used here.

Relationship of Protein Bound Water as Calculated by Nuclear Magnetic Resonance-Relaxation to Water Activity

As solutes are dissolved in water, the colligative properties of water (e.g., freezing point depression) are changed. These changes are the result of decreased vapor pressure, or viewed another way, the solute-solvent interactions lower the activity of water (a_w). According to Raoult's Law (33), a_w can be defined as follows:

$$a_w = \frac{n_2}{n_2 + n_1} \quad [9]$$

where n_1 and n_2 are the number of moles of solute and solvent, respectively. Equation [9] works well for small molecules such as simple sugars and salts, but complications arise in attempting to predict the contribution of proteins to a_w . As a first approximation, we have used Equation [4] along with the following expression (Equation [10]) to evaluate a_w for each protein concentration studied (17):

$$a_w = 1 - \bar{v}_w ap \quad [10]$$

Here \bar{v}_w is obtained from the isotropic tumbling model with $S = 1$, and ap is defined in Equation [4] with c in g/ml. Taking the hydration values from Tables 1 and 2 as a starting point, and the calculated virial coefficients, the a_w contribution for proteins can be modeled for any theoretical concentration. The extrapolated values for β -lactoglobulin and caseins are shown in Figure 8.

As can be seen in Figure 8, even at a concentration of 300 mg/ml, β -Lg A cannot suppress the activity of water below .96 under any of the environmental conditions studied. Although the hydration of β -Lg A at pH 4.65 and 2°C is increased to .0301 gH₂O/g protein due to octamer formation, the low value of the

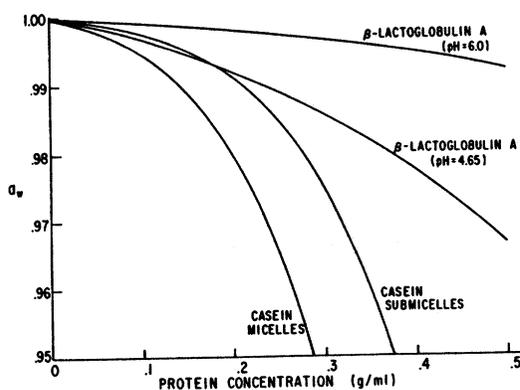


Figure 8. Theoretical curves for water activity (a_w) from .95 to 1.0 versus protein concentration in grams per milliliter for the milk protein at 2°C calculated from hydration values and Equations [4] and [10].

second virial coefficient, namely .0008 ml/mg, does not allow effective suppression of water. Hence, at relatively neutral pH, β -Lg A alone would not be an effective additive for suppression of the a_w . It is important to note that in these model calculations only the isotropic hydrations ($S = 1$, Table 2) were employed. When anisotropic hydration (Table 3) was used, the equations overemphasize the contribution of protein-bound water to a_w suppression. For example, for micelles at 2°C the predicted concentration required for suppression to .95 is only 6% protein. Such predictions are not in accord with empirical observations on milks (33). Thus, more appropriate estimations of effects of protein on a_w are obtained with $S = 1$.

As expected, a greater \bar{v} for the micellar form of casein is accompanied by greater suppression of water activity. Figure 8 shows micellar casein to exhibit a greater a_w suppression than submicellar casein over the entire range of protein concentrations. By extrapolation of those data, for casein micelle concentrations of 300 mg/ml (10 times that which normally occurs in milk) and a temperature of 2°C, a_w is .943. This activity level is well below the minimum needed to support growth of many bacteria, including *Lactobacillus viridescens*, some strains of *C. botulinum*, *E. coli*, *Salmonella*, and *Pseudomonas*

fluorescens (33). Theoretically, a 10:1 concentrate of skim milk at 4°C would retard bacterial growth by virtue of a_w suppression due only to protein, without even considering suppression resulting from elevated salt and lactose concentrations. Similar reduction of water activity with the submicelles alone does not occur. Apparently, some forms of protein can suppress water activity, but only under specified conditions which include the virial coefficient of the protein and its charge to mass ratio (17). In this case it may also be related to the formation of colloidal complexes with the concomitant trapping of water. Hence, all environmental conditions leading to structural changes must be taken into account when evaluating the optimum contribution of a protein to a_w suppression.

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