# Comparison of Hydration Behavior of Bovine and Caprine Caseins As Determined by Oxygen-17 Nuclear Magnetic Resonance: pH/pD Dependence

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The $^{17}O$ NMR transverse relaxation rates ($R_2$, $s^{-1}$) of water in bovine and two caprine casein solutions were studied at 21 °C with pH/pD values of 4.81, 6.95, and 8.16. The dependence of $R_2$ was markedly nonlinear for all three proteins. Bovine and caprine caseins differ in the magnitude and the sign of the $B_0$ virial coefficient, which is attributable to protein composition and structure. The composition of bovine casein and caprine casein high in $\alpha_{s1}$-casein solutions at pD 4.81 is reflected in the negative sign of the $B_0$ virial coefficient and appears to be correlated with satisfactory aggregation (coagulation) properties. In acid-treated caprine casein solutions with low $\alpha_{s1}$-casein content, the positive sign of the $B_0$ virial coefficient suggests structural environments which are resistant to self-association. The $B_0$ values at pD 8.16 are also negative, indicative of unfolding leading to self-association. Analysis of the results also reveals marked differences in hydration behaviors, determined primarily by compositional and structural differences. The hydration of all three caseins is increased at acid and at alkaline pH/pD (pD 4.81 and 8.16), with minima at neutral pH/pD (pD 6.95). The hydration sensed by the $^{17}O$ NMR experiments on casein, however, represents internal "trapped" water which serves to report on environmental changes. Enhanced self-association (prior to precipitation) of bovine and caprine caseins at acid conditions (pD 4.81) may account for the increased hydration. Alkali treatment (pD 8.16) may lead to swelling and possibly denaturation, all of which may enhance hydration. Our results demonstrate the feasibility of monitoring functional coagulation properties of bovine and caprine milks by NMR relaxation measurements.

**Keywords:** *NMR, $^{17}O$; water binding; pH/pD effects; bovine casein; caprine casein; caprine $\alpha_{s1}$-casein*

## 1. INTRODUCTION

Proteins are amphoteric in nature because of the presence of both acidic and basic side chains on the protein molecules; that is, they can possess either a net positive or a net negative charge in solution, reflecting the prevailing environment. These changes are dependent upon the extent of dissociation of these groups and the character and number of any bound ions. The common methods for investigating the electrochemical nature of milk proteins are their titration curves and electrophoretic behavior (Thompson, 1970). In this context, the shape of the titration curve not only is a function of the primary structure of the protein but may reflect either its degree of association or its configuration. For example, in the case of the $\alpha_{s1}$-casein, it was demonstrated that, while ionic strength influences the character of the coagulation below pH 6.4, the titration curves indicated that all of the ionizable groups, in whatever form the $\alpha_{s1}$-casein occurs, are accessible and completely reversible to proton exchange (Ho and Waugh, 1965). The agreement between the ionizable groups as determined from the titration curves and that calculated from the primary structure is good (Swaisgood, 1983).

The presence of attractive and repulsive interactions among proteins in solution can perturb local water motions and affect water "binding" (Kumosinski and Pessen, 1982). Hence, protein hydration should be affected. Therefore, it is important to be able to quantitate these charge, dipolar, polar, and nonpolar interactions and to determine how they influence the hydration behavior of proteins.

Nuclear magnetic resonance (NMR) relaxation techniques provide useful, nondestructive methods for studying the amount and the mobility of "bound" water (i.e., the fraction of water that is significantly perturbed by the protein) and the effect of various intermolecular interactions on this water (Derbyshire, 1982; Bryant and Halle, 1982; Pessen and Kumosinski, 1985; Piculell and Halle, 1986). $^2H$ NMR has been used to probe water interactions in several aqueous protein systems including bovine milk proteins such as $\beta$-lactoglobulin A (Kumosinski and Pessen, 1982) and caseins (Kumosinski et al., 1987; Farrell et al., 1989).

The purpose of this study is to investigate further the relationship between protein−protein interactions and protein hydration in bovine and caprine casein solutions at various values of pH/pD on the basis of $^{17}O$ NMR relaxation measurements. Our study is also aimed at relating the NMR observations to comparison of the functional coagulation properties of bovine and caprine caseins in dairy food systems.

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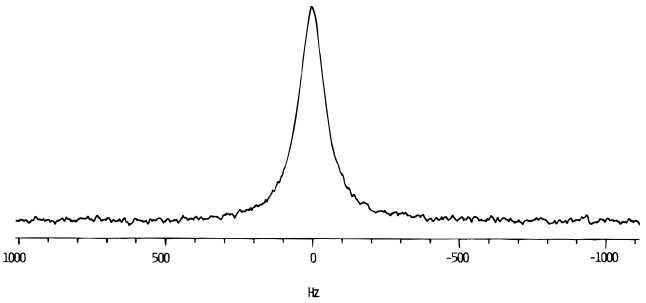


**Figure 1.** $^{17}O$ NMR spectrum of bovine casein (2.72% w/v) in $D_2O$ at 21 ± 1 °C and pD 4.81.

## 2. MATERIALS AND METHODS

**2.1. Materials.** All reagents used were of analytical grade or ACS certified. $D_2O$ (99.8% D), DCl, and NaOD (minimum 99.0% D) used were purchased from Sigma Chemical Co. (St. Louis, MO).

**2.2. Sample Preparation.** The preparation of whole caseins from bovine and caprine milk was as previously described (Mora-Gutierrez et al., 1995); the caprine whole caseins were selected as yielding high and low levels of the $\alpha_{s1}$-casein component as determined by reversed-phase high-performance liquid chromatography (RP-HPLC) (Mora-Gutierrez et al., 1991). Note the terms high and low represent 2.70 and 0.12 g/L, respectively. This contrasts with bovine casein, which contains on the average 10 g/L. The total casein contents of both milks average 25−30 g/L.

Protein samples for NMR measurements were prepared by mixing the appropiate amount of lyophilized whole casein in deuterium oxide ($D_2O$). The initial sample pH was 7.0; it was adjusted (±0.01 pH unit) by slowly adding DCl or NaOD with a microsyringe with continuous stirring. Conversion to pD values was made according to the relation pD = pH + 0.4 (Covington et al., 1968), where pH is the pH meter reading for a $D_2O$ solution with the electrode calibrated in standard $H_2O$ buffers. Samples were transferred to 10 mm high-resolution NMR tubes (Wilmad, Buena, NJ). All measurements were completed within 12 h after sample preparation. Such measurements were carried out in duplicate at 21 ± 1 °C.

**2.3. $^{17}O$ NMR Spectroscopy.** A Varian XL-200 spectrometer (Varian Associates, Palo Alto, CA) operating at 4.7 T was used for $^{17}O$ NMR measurements on bovine and caprine casein samples. The number of scans required for a good signal-to-noise ratio (>100:1) in the $^{17}O$ NMR spectra of bovine and caprine casein samples was about 1000. Further details concerning the NMR experimental conditions are given in the figure captions.

Figure 1 shows the Fourier transform spectra of a 2.72% (w/v) bovine casein dispersion in $D_2O$ at pD 4.81. Line widths were measured at half-height of each spectrum. To correct for any residual magnetic field inhomogeneity, the "net" line broadening ($\Delta\upsilon_B$) was calculated by subtracting the line width of liquid $D_2O$ ($\Delta\upsilon_{free}$) from that of the sample ($\Delta\upsilon_{obs}$). The net or differential transverse relaxation rates ($\Delta R_2$, $s^{-1}$) were then calculated from the line widths according to the standard formula:

$$\Delta R_2\ (s^{-1}) = \pi\Delta\nu_B\ (s^{-1}) = \Delta T_2{}^{-1}\ (s) \qquad (1)$$

**2.4. Data Analysis.** The dependence of the observed NMR relaxation rates on protein concentrations was fit with an iteractive nonlinear regression program (SYSTAT v. 5.1) which employed the Quasi-Newton algorithm to obtain confidence intervals for the iterated parameters. The program was run on a Macintosh II microcomputer (Apple Computer, Inc., Cupertino, CA), with a 68020 CPU and a 68882 (16-MHz) mathematical coprocessor. This program minimizes the standard deviation points from the curve, also known as the root mean square (RMS), where the RMS is defined as

$$\text{RMS} = \{[\sum(R_{2obs} - R_{2calc})^2]/(\text{no. of data points} - \text{no. of fitting parameters})\}^{1/2} \qquad (2)$$

where $R_{2obs}$ and $R_{2calc}$ are the observed and calculated relaxation rates, respectively. RMS values were normalized to be within at least 5% error of the fit for all the data.

## 3. RESULTS AND DISCUSSION

**3.1. $^{17}O$ Nuclear Magnetic Resonance.** The majority of NMR studies have used the $^1H$ nucleus of water as a probe. However, difficult problems with the interpretation of the $^1H$ NMR relaxation data in macromolecular systems were discovered; the primary concern is with the relative influence of the various relaxation mechanisms which contribute to the line width of the water peak in the $^1H$ NMR spectra of such complex systems (Kalk and Berendsen, 1976; Edzes and Samulski, 1978; Koenig et al., 1978). The two relaxation mechanisms that contribute significantly to the water proton line broadening are (1) the cross-relaxation between the bulk water protons and protons of the macromolecule and (2) the proton exchange between distinct states of water, i.e. "bound" and "free" water.

Therefore, recent interest (Denisov and Halle, 1995) in measuring water binding and hydration of macromolecules has been directed to quadrupolar nuclei ($I > 1/2$). Nuclei with spin $I > 1/2$ possess electric quadrupole moments, and their relaxation behavior is usually dominated by the interaction of the nuclear quadrupole with the electric field gradient at the nucleus (Halle and Wennerström, 1981). This is the case for $^2H$ ($I = 1$) and $^{17}O$ ($I = 5/2$). Both the $^2H$ and the $^{17}O$ nuclei avoid the problem of cross-relaxation. However, the $^2H$ nucleus is still affected by chemical exchange whereas, except for a relatively narrow pH range around neutrality, the $^{17}O$ is not influenced by chemical exchange (Halle et al., 1981). Halle et al. (1981) list two other advantages of probing the $^{17}O$ nucleus: (1) the strong quadrupolar interaction leads to large relaxation effects, thus allowing studies at rather low concentrations; (2) the intramolecular origin of the electric field gradient at the water oxygen nucleus makes the quadrupolar interaction virtually independent of the molecular environment, which greatly facilitates interpretation of the relaxation data.

**3.2. Model for NMR Data Interpretation.** Analysis of NMR relaxation data is strongly model-dependent. For water populations undergoing isotropic motions in bovine and caprine casein solutions in deuterium oxide, a two-state model with fast exchange between weakly bound and free water molecules is appropiate (Derbyshire, 1982); a linear dependence of relaxation rates on the protein-to-water ratio is predicted by this model if no additional contributions are present (Zimmerman and Brittin, 1957; Derbyshire, 1982). This relationship results from the population weighted average between bound water relaxation rates ($R_B$) and free water relaxation rates ($R_F$), which is expressed as

$$R_{obs} = P_B R_B + P_F R_F \qquad (3)$$

where $P_B$ and $P_F$ are fractions of bound and free water in the system, respectively, and $R_{obs}$ is the measured water relaxation rate of the samples at various hydration levels.

If $C_p$ is protein concentration in milligrams of protein per milligram of water and $n_H$ is the protein hydration in milligrams of bound water per milligram of protein, then

$$P_B = n_H C_p \quad (4)$$

and, since $P_F = 1 - P_B$, eq 4 may be rewritten as

$$R_{obs} = (R_B - R_F)P_B + R_F = n_H(R_B - R_F)C_p + R_F \quad (5)$$

with $R_{obs}$, $R_B$, and $R_F$ being either the longitudinal ($R_1$) or transverse ($R_2$) relaxation rates.

Equation 6 predicts a linear relationship between $R_{2obs}$ and the macromolecular concentration $C_p$ (Pessen and Kumosinski, 1985):

$$R_{2obs} = R_{2F} + n_H \Delta R C_p \quad (6)$$

$n_H$ is the protein hydration and $\Delta R = R_{2B} - R_{2F}$. However, a protein molecule in solution is influenced by its environment, which includes other protein molecules surrounding it. The nonideality of a protein solution is the result of such environmental interactions; specifically, nonideality is caused by long-range electrostatic interactions, charge fluctuations (in the absence of salt), dipole moments, multipoles, and fluctuations in the distribution of the protein protons [changes in both the number and configuration of bound protons (Kirkwood and Shumaker, 1952)]. There are both repulsive and attractive forces associated with such processes, and the virial coefficients are, therefore, sensitive to the protein charge, as well as its structure. However, charge−charge interactions are almost always repulsive. Therefore, the activity of a protein ($a_p$) in solution must be considered. Protein activity is related to its concentration, $C_p$, by the activity coefficient, $\gamma_p$:

$$a_p = \gamma_p C_p \quad (7)$$

The activity coefficient can be determined from the virial expansion

$$d \ln \gamma_p / dC_p = 2B_0 + 3B_2 C_p + ... \quad (8)$$

where the $B_i$ quantities are the virial coefficients of the protein activity. When this virial expansion is applied in conjunction with the two-site model with fast exchange, one equation results for bovine and caprine casein solutions at various pH/pD values:

$$R_{2obs} - R_{2F} = n_H(R_{2B} - R_{2F})C_p \exp(2B_0 C_p + 2B_{0.5} C_p^{0.5} + 0.667 B_{1.5} C_p^{1.5} + 1.5 B_2 C_p^{2} + ...) \quad (9)$$

where $n_H$ is the Scatchard hydration and all other variables are as defined previously. The $B_0$ virial coefficient reflects interactions related to the net average charge on the proteins ($Z$), a preferential interaction term, and a contribution from the protein excluded volume ($\bar{v}_p$). The $B_2$ virial coefficient represents attractive forces caused by higher fluctuating multipoles that modulate protein−protein interactions. Under salt-free, "isoionic" conditions it has been shown that the protein light scattering data exhibit a square root ($C_p^{1/2}$) dependence on protein concentration (Timasheff et al., 1957; Kronman and Timasheff, 1959). In this instance, another virial coefficient, $B_{1/2}$, would need to be added to the expansion. The $B_{1/2}$ coefficient of the $C_p^{1/2}$ term is caused by charge fluctuations of residues at the protein surface. Futhermore, charge fluctuations become dominant in the dilute concentration range, in the absence of salt or other ionizable species. Addition of salt or base to the solution results in a charge-screening effect which practically eliminates the $B_{1/2}$ term (Kirkwood and Shumaker, 1952; Kronman and Timasheff, 1959). Doty and Steiner (1952) proposed a different model based on the virial coefficients of Boltzmann that appeared to fit the light scattering data quite well for bovine serum albumin (BSA), but this model may suffer in that it ignores protein ionization and preferential binding (Kronman and Timasheff, 1959; Arakawa and Timasheff, 1982).

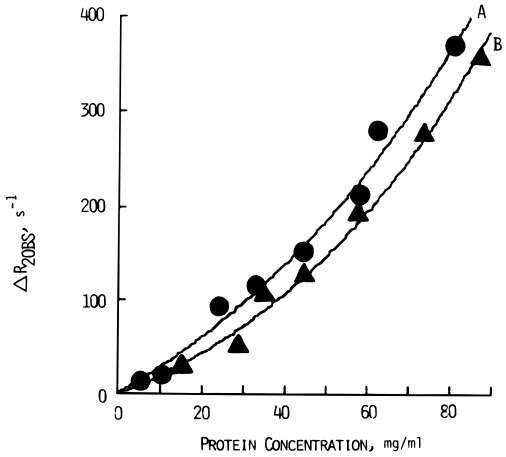


**Figure 2.** Dependence of the 27.1 MHz $^{17}O$ NMR transverse relaxation rates on protein concentration for (A) bovine casein and (B) caprine casein low in $\alpha_{s1}$-casein in the absence of salt at 21 ± 1 °C and pD 6.95. Spectra were recorded with single radio frequency pulses of 19 $\mu$s pulsewidth (90° flip angle), in the presence of broadband proton decoupling at 200.05 MHz, 1024 scans were recorded with a recycle time of 0.5 s, and data were processed with 5.0 Hz line broadening (by exponential multiplication).

**3.3. Influence of Protein Activity on $^{17}O$ NMR Transverse Relaxation Rates.** Figure 2 represents the observed $^{17}O$ NMR transverse relaxation rates, $\Delta R_{2obs}$ ($s^{-1}$), as a function of protein concentration (milligrams per milliliter) for bovine (A) and caprine casein low in $\alpha_{s1}$-casein (B) in $D_2O$ at pD 6.95 in the absence of salt. In both cases, the relaxation rates appear to increase linearly with protein concentration up to approximately 12 mg/mL, indicating that the protein activity is small in this region. Above this point, the relaxation rate increases nonlinearly with protein concentration, indicating that protein activity coefficients have appreciable values. Under the same conditions a less marked (but statistically significant) deviation from linearity was observed for caprine casein high in $\alpha_{s1}$-casein (Figure 3). The results obtained from the nonlinear regression analysis of the NMR relaxation data using eq 9 are presented in Tables 1 and 2.

At all pD values tested, in the protein concentration range from 0 to 90 mg/mL (Figures 2−7) no coefficients other than $B_0$ were needed to fit the NMR relaxation data (no significant decrease in RMS). The absence of nonzero $B_{n/2}$ terms is most likely due to the charge-screening effect resulting from the base/acid required for the pH/pD adjustment. The positive sign of the calculated virial coefficients (Table 1) indicates that protein−protein repulsions dominate in bovine and caprine casein solutions at pD 6.95 in the absence of salt. The presence of these repulsive forces could be the result of the contribution of its surface charges, par-

**Table 1. Calculated Virial Coefficients $B_0$[a] from Nonlinear Regression Analysis of $^{17}O$ NMR Transverse Relaxation Data for Bovine and Caprine Caseins in $D_2O$ at 21 ± 1 °C Using Equation 9**

| | pD | | |
|---|---|---|---|
| casein | 4.81 | 6.95 | 8.16 |
| bovine | −0.00469 ± 0.0002 | 0.00356 ± 0.0002 | −0.00220 ± 0.0001 |
| caprine | | | |
| high $\alpha_{s1}$-casein | −0.00434 ± 0.0002 | 0.00077 ± 0.0001 | −0.00292 ± 0.0002 |
| low $\alpha_{s1}$-casein | 0.00417 ± 0.0003 | 0.00481 ± 0.0002 | −0.00116 ± 0.0004 |

[a] In mL/mg. The protein concentration was in mg of protein/mL of solvent.

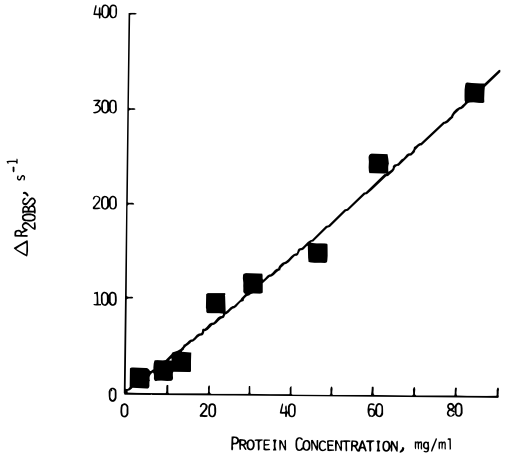


**Figure 3.** Dependence of the 27.1 MHz $^{17}O$ NMR transverse relaxation rates on protein concentration for caprine casein high in $\alpha_{s1}$-casein in the absence of salt at 21 ± 1 °C and pD 6.95. The same conditions as in Figure 2 were used.

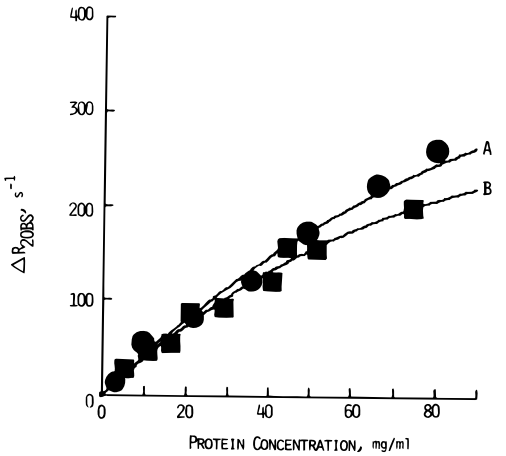


**Figure 4.** Dependence of the 27.1 MHz $^{17}O$ NMR transverse relaxation rates on protein concentration for (A) bovine casein and (B) caprine casein high in $\alpha_{s1}$-casein in the absence of salt at 21 ± 1 °C and pD 8.16. The same conditions as in Figure 2 were used.

**Table 2. Calculated Hydration Products $n_H\Delta R$[a] from Nonlinear Regression Analysis of $^{17}O$ NMR Transverse Relaxation Data for Bovine and Caprine Caseins in $D_2O$ at 21 ± 1 °C Using Equation 9**

| | pD | | |
|---|---|---|---|
| casein | 4.81 | 6.95 | 8.16 |
| bovine | 6.86 ± 0.042 | 2.60 ± 0.023 | 4.33 ± 0.028 |
| caprine | | | |
| high $\alpha_{s1}$-casein | 5.37 ± 0.041 | 3.36 ± 0.018 | 4.15 ± 0.036 |
| low $\alpha_{s1}$-casein | 3.49 ± 0.038 | 1.81 ± 0.022 | 3.55 ± 0.052 |

[a] In mL $mg^{-1}$ $s^{-1}$. See footnote *a* of Table 1.

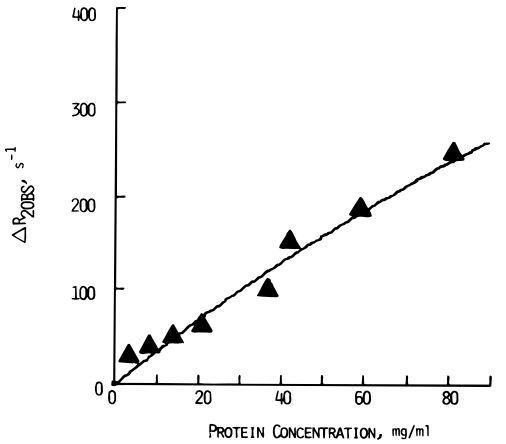


**Figure 5.** Dependence of the 27.1 MHz $^{17}O$ NMR transverse relaxation rates on protein concentration for caprine casein low in $\alpha_{s1}$-casein in the absence of salt at 21 ± 1 °C and pD 8.16. The same conditions as in Figure 2 were used.

ticularly charged phosphate groups mainly located on the surface of the casein components; the region between pH 6.0 and 7.0 is that in which the phosphoserine residues of the protein are expected to titrate, and this is likely to be a major factor in determining these repulsive electrostatic forces. Indeed, at this pH/pD and in the low ionic strength conditions studied here, all of the major phosphorylated caseins ($\alpha_{s1}$-, $\alpha_{s2}$-, and $\beta$-casein) exhibit their lowest degrees of self-association (Schmidt, 1982).

Inspection of the $B_0$ values from Table 1 for caprine caseins at pD 6.95 shows a significantly higher value for the caprine casein low in $\alpha_{s1}$-casein. It is possible that the larger extent of electrostatic repulsions exhibited by the caprine casein low in $\alpha_{s1}$-casein favors a more open structure at pH values between 6.0 and 7.0. Such differences, if they persist in products, may affect substantially optimum "curd firmness". The firmness of caprine milk gels increases dramatically in the order caprine low in $\alpha_{s1}$-casein < caprine high $\alpha_{s1}$-casein (Ambrosoli et al., 1988).

The nonlinear regression analysis of the protein concentration dependences of $^{17}O$ NMR transverse relaxation rates at pD 8.16 in the absence of salt (Figures 4 and 5) yields negative values for the second virial coefficients for all of the caseins, $B_0$ (Table 1). These negative virial coefficients at pD 8.16 may indicate increased self-association due to increased favorable charge−charge attractions. In the case of caprine casein low in the $\alpha_{s1}$-component, the $B_0$ value is much lower ($B_0 = -0.00116$ mL/mg) than in the case of caprine casein high in $\alpha_{s1}$-casein ($B_0 = -0.00292$ mL/mg), implying that for caprine casein the degree of self-association is weaker in the presence of low amounts of the $\alpha_{s1}$-casein component and correspondingly higher amounts of $\alpha_{s2}$-casein. However, caprine casein high in $\alpha_{s1}$-casein may aggregate more than bovine casein, which is greatest in $\alpha_{s1}$ content but has a $B_0$ of −0.00220, which lies between the two caprine caseins (as one can see from the downward curvature of the $\Delta R_2$ dependence at higher protein concentrations, Figure

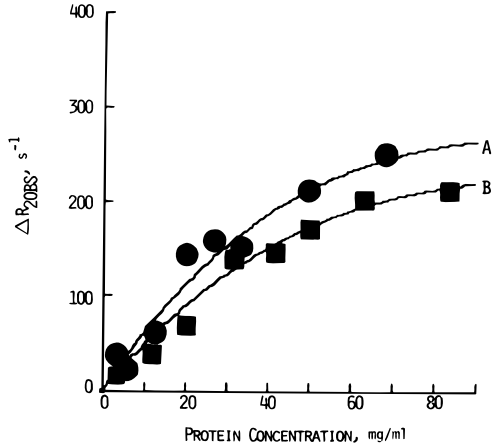


**Figure 6.** Dependence of the 27.1 MHz $^{17}O$ NMR transverse relaxation rates on protein concentration for (A) bovine casein and (B) caprine casein high in $\alpha_{s1}$-casein in the absence of salt at 21 ± 1 °C and pD 4.81. The same conditions as in Figure 2 were used.

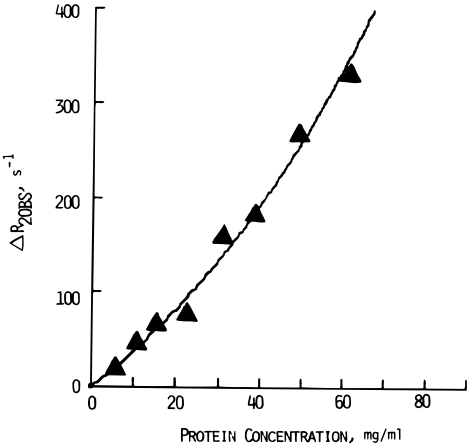


**Figure 7.** Dependence of the 27.1 MHz $^{17}O$ NMR transverse relaxation rates on protein concentration for caprine casein low in $\alpha_{s1}$-casein in the absence of salt at 21 ± 1 °C and pD 4.81. The same conditions as in Figure 2 were used.

4B). Here the mixed associations appear to produce a larger aggregate, depending in part on the ratios of casein monomers present and, perhaps, on the degree of glycosylation of caprine $\kappa$-casein. A smaller but significant tendency to aggregate was also observed for caprine casein low in $\alpha_{s1}$-casein (Figure 5) as compared to that of bovine casein (Figure 4A). Increased association of caprine caseins might be ascribed to a slightly lower net charge on these milk proteins. Evidence supporting this hyphothesis was given (Mora-Gutierrez et al., 1995) when it was shown that caprine whole caseins characterized by high and low content of the $\alpha_{s1}$ component have lower net protein charges (−12) than bovine whole casein (−16). Moreover, it should be noted that at very high pH values (i.e., pH 11−12) the caseins are known to be in their "monomeric" state (Waugh and von Hippel, 1956; McKenzie and Wake, 1959), suggesting that electrostatic forces between casein molecules are weakened by the conversion of positively charged groups to neutral amines or guanido groups.

Caseins, by definition, are phosphoproteins that precipitate from skim milk at pH 4.6 and 20 °C. At pH values below the p*I*, where carboxyl groups are titrated, lysine and arginine would remain protonated and, therefore, are primarily responsible for water−protein interactions. Figures 6 and 7 show the $^{17}O$ transverse relaxation rates, $\Delta R_2$, for bovine and caprine caseins in $D_2O$ at pD 4.81 in the absence of salt. The behaviors of the bovine and caprine caseins high in $\alpha_{s1}$-casein relaxation rates have a marked nonlinear concentration dependence at this acid pH/pD (Figure 6). Parallel studies of caprine casein low in the $\alpha_{s1}$ component exhibited also a marked nonlinear behavior (Figure 7) but quite different from those of Figure 6. Virial coefficients of protein activity (Table 1) were obtained from the nonlinear regression analyses of the NMR data presented in Figures 6 and 7 according to eq 9.

For solutions of bovine and caprine casein high in $\alpha_{s1}$-casein at pD 4.81 (Figure 6), the $B_0$ terms exhibit a negative sign, which indicates the presence of attractive interactions between protein molecules. The value required for the second virial coefficient, $B_0$, to produce a good fit of the $^{17}O$ water NMR data of bovine and caprine casein concentrations at pD 4.81 presented in Figure 6 ($B_0 = -0.00469$ and $-0.00434$ mL/mg, respectively) suggests the presence of relatively stronger attractive forces among these caseins. In bovine milk, the effect of pH on coagulation and curd firmness is well-known (Okigbo et al., 1985a−c). The magnitude of these attractive forces probably contributes to the increased curd firmness observed in acid-coagulated milk of cows (Emmons et al., 1960); this hypothesis may also help to explain the suitability of caprine milk high in $\alpha_{s1}$-casein for cottage cheese and yogurt making, as it would produce a firmer curd.

The positive $B_0$ virial coefficient for caprine casein low in $\alpha_{s1}$-casein at pD 4.81 in the absence of salt (Table 1) is attributed to increased charge−charge (repulsive) interactions which should lead to a decrease of protein self-association into polymers. Although the positive $B_0$ virial coefficient for caprine casein low in $\alpha_{s1}$-casein (0.00417 mL/mg) is large, the magnitude of such strong repulsive interactions may become significant if these charges are partially or completely buried inside the protein where the dielectric constant is lower. In such cases, each of the buried charges will destabilize the native protein structure to the order of 20 kcal/mol (Lumry and Biltonen, 1969). The presence of strong charge−charge (repulsive) electrostatic forces (positive $B_0$ values) in caprine casein low in $\alpha_{s1}$-casein induced by changes in pH/pD ($B_0 = 0.00481$ and 0.00417 mL/mg at pD values of 6.95 and 4.81, respectively) indicates the potential for poor gel formation of low-type caprine milk during the manufacture of cheese and yogurt products.

**3.4. Bovine and Caprine Casein Hydration.** Either $n_H$ or $\Delta R$ may be responsible for the observed effect of pH/pD on the bovine and caprine casein hydration product $n_H\Delta R$ (Table 2). At the lower and higher pD values, the bovine and caprine casein hydration product $n_H\Delta R$ (Table 2) increased relative to the values at pD 6.95 which had the lowest hydration products (Table 2). From the hydration products of Table 2 an apparent hydration value $n_H$ may be calculated (Table 3). The changes in $n_H$ exactly parallel those of the hydration product. However, the size of $n_H$ deserves specific attention. The average hydration in Table 3 is 0.0065 g of water/g of protein. These values are quite close to the hydration values derived by $^2H$ NMR (Kumosinski et al., 1987). The hydration numbers were calculated by assuming the correlation times found for bovine casein. The magnitude of these numbers and their correspondence with the $^2H$ NMR values indicate that the $^{17}O$ experiments sense the same "window" on the casein that other NMR techniques reveal; i.e., the

**Table 3. Hydration $n_H$[a] Estimates of Bovine and Caprine Caseins[b]**

| | pD | | |
|---|---|---|---|
| casein | 4.81 | 6.95 | 8.16 |
| bovine | 0.00579 | 0.00356 | 0.00962 |
| caprine | | | |
| high $\alpha_{s1}$-casein | 0.00670 | 0.00510 | 0.00694 |
| low $\alpha_{s1}$-casein | 0.00826 | 0.00255 | 0.00982 |

[a] g of water/g of protein; assuming $\tau_c = 56$ ns for bovine casein submicelles (Kakalis et al., 1990). [b] From $^{17}O$ NMR data at 21 ± 1 °C and at pD 4.81, 6.95, and 8.16; according to a two-state, isotropic model (Mora-Gutierrez et al., 1995).

water observed is internal or trapped water associated with submicellar particles (Kumosinski et al., 1987; Farrell et al., 1989). This conclusion is borne out by the recent $^{17}O$ NMR experiments by Denisov and Halle (1995). The latter workers were able to mathematically separate the contributions to relaxation of $^{17}O$ by various classes of exchangeable water through field dependency studies. For bovine pancreatic trypsin inhibitor (BPTI) the classes of water molecules included 4 internal (trapped) water molecules, 250 surface water molecules, and 2500 protein-influenced bulk water molecules. However, the four internal water molecules contribute disproportionately to the relaxation. Although we observe a weighted average for the relaxation and are studying a more complex protein system in our work, internal or "trapped" water undoubtedly dominates the experiment. This dominance is true for the casein aggregates, which provide many internal cavities and surface pockets for water. Therefore, our experiments report changes undergone by this trapped water and not water transiently bound to surface charges. Kumosinski et al. (1994) proposed an apparent molecular mechanism to account for the nature of the trapped water by comparison of casein 3D models and small-angle X-ray scattering data. Regardless of the exact positioning of this trapped water, the $^{17}O$ NMR data describe changes with environmental conditions. We will therefore discuss how the changes in our "reporter hydrations" correlate with changes in casein associations.

It has been demonstrated that bovine $\alpha_{s1}$-casein exists primarily as a monomer at pH 6.6 and 8.0 and ionic strength of 0.01 (Schmidt, 1982). In contrast, mixed associations between $\alpha_{s1}$- and $\kappa$-casein result in polymers under these conditions (Slattery and Evard, 1973) and $\beta$-casein is at least a dimer (Schmidt, 1982). The degree of self-association of whole caseins under these conditions (low ionic strength and 21 °C) is probably intermediate in nature. At ionic strengths of 0.1, small angle X-ray and neutron scattering consistently show that caseinates in the absence of $Ca^{2+}$ are associated and have a loose exterior and a more dense core (Farrell et al., 1990) under conditions of maximum association. At 21 °C both core and loose exterior would be highly porous and associated and would exhibit trapped water, which is our reporter as noted above.

As the pD of the casein solution is raised to 8.16, further hydration occurs for all caseinates (Table 3). Two factors are at work at higher pH/pD: according to the $B_0$ values of Table 1 there are increased attractive forces which favor casein association; alkali treatment could generate more aperiodic secondary structures (Swaisgood, 1983). Thus, increased hydration can be accounted for in the increased aggregation following protein denaturation. Such increased hydration accompanying alkaline denaturation was observed for $\beta$-lactoglobulin (Pessen et al., 1985). Our results most likely indicate that the self-association of casein into larger "polymers", which is observed in the absence of salt at low and high pH/pD values, is correlated with an increased amount of internal, but exchangeable, trapped water. This is true even for the low $\alpha_{s1}$-caprine casein even though the $B_0$ value is indicative of repulsive rather than attractive forces. The dependence of $\Delta R_{2obs}$ on protein concentration for bovine and caprine caseins in $D_2O$ at pD values of 4.81 and 8.16 (Figures 4−7) may thus be rationalized as the effect of pH/pD on the protein aggregation state and perhaps denaturation, which lead to increased internal hydration (Table 3).

The results presented here, which are based on experiments with solutions of bovine and caprine caseins in the absence of salt, appear at first glance not to be readily comparable with the self-association process observed on bovine caseins and carried out with $CaCl_2$ solutions (Kumosinski et al., 1987). Here, the $^{17}O$ NMR transverse relaxation rates of bovine and caprine casein solutions in $D_2O$ were analyzed by assuming that the magnitude of the net charge on the protein molecule as affected by pH/pD contributes to the $^{17}O$ nuclear spin relaxation. The variation with pH/pD of the protein net charge contribution is reflected in the sign of the virial coefficient of protein activity ($B_0$) for bovine and caprine caseins (Table 1). However, protonation of soluble casein by reducing pH has a parallel effect to calcium aggregation. Addition of acid ultimately causes the precipitation of casein molecules. Preceding total precipitation, aggregation occurs. In the preparation of acid caseinates the environment affects curd size and draining (Jablonka and Munro, 1985). Prior to coagulation and precipitation, casein particles begin to aggregate and then form the curd. So there is a formal physical analogy between micelle formation (aggregation due to $Ca^{2+}$) and acid-induced aggregation: both represent a form of charge neutralization by $Ca^{2+}$ or $H^+$. In this work, acidification leads to higher hydration as casein aggregation occurs prior to complete precipitation. The temperature of the experiment, 21 ± 1 °C, prevents precipitation at pD 4.81. It is interesting to speculate that the low $\alpha_{s1}$ caprine caseins with greatest internal hydration and a positive $B_0$ value at pD 4.81 (Tables 3 and 1, respectively) might well yield softer curd cheeses, perhaps because of their lower degree of aggregation and higher water binding. The marked differences in hydration behavior between the two caprine caseins are determined primarily by compositional differences. Thus, it can be said that the differences in the caprine casein high in $\alpha_{s1}$-casein at pH/pD 6.95 (Tables 2 and 3) may be due to the greater amount of $\alpha_{s1}$-casein, whereas in the low $\alpha_{s1}$-casein, $\alpha_{s2}$- is substituted. This protein contains a higher percent of positive charge (Eigel et al., 1984). The increase of the hydration product $n_H\Delta R$ in the absence of salt at pD 6.95 for caprine casein high in $\alpha_{s1}$-casein seems to indicate that the water molecules could bind to the phosphorylated and other charged sites in the casein. However, as noted above Kumosinski et al. (1994) have suggested that these more tightly bound (but exchangeable) water molecules more likely occur with internal cavities, and this correlates well with the data of Denisov and Halle (1995). The changes observed here could be more related to $\alpha_{s1}$−, $\alpha_{s2}$−$\kappa$-casein interactions leading to complexes with altered associative properties.

## 4. CONCLUDING REMARKS

The results presented here have shown that $^{17}O$ NMR studies of water relaxation ($R_2$) can serve as a reporter on the nature and degree of association of casein monomers under a variety of conditions. However, $^{17}O$ NMR relaxation measurements, combined with $^{13}C$ NMR spectroscopy, would provide more detailed information about the molecular structure and dynamics of caseins and can offer new insight into processes that involve dissociation into subunits and protein denaturation such as thermal denaturation (Morr, 1985) and protein gelation (Schmidt and Morris, 1984).

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