Sugar−Casein Interaction in Deuterated Solutions of Bovine and Caprine Casein As Determined by Oxygen-17 and Carbon-13 Nuclear Magnetic Resonance: A Case of Preferential Interactions

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^{17}O NMR spectroscopy and ^{13}C NMR spectroscopy have been used to study the mechanism of interaction of sugars with bovine and caprine caseins in D_2O. The ^{17}O NMR relaxation results showed in all cases an increase in water of hydration, as a result of added sugar; this was predominantly associated with "trapped" water in the caseins. Analysis of the virial coefficients, obtained from the ^{17}O relaxation data, suggested that preferential interactions occur in the sugar−protein solutions. This could be the result of either sugar binding or a solute−solute thermodynamic effect, preferential hydration. The addition of sugars to deuterated solutions of bovine casein and caprine casein high in α_{s1}-casein had little or no effect on either line width or chemical shilfts of the ^{13}C NMR spectra of these milk proteins. ^{13}C NMR studies of sucrose, at various concentrations (100−300 mM) in the presence of caprine casein high in α_{s1}-casein, showed no changes in either chemical shifts or T_1 values. This indicates that the sugar molecules tumble isotropically and therefore neither bind to the protein nor affect viscosity in the protein−sugar studies. All of these data suggest that the preferential exclusion of the sugar from the domain of the caseins results in preferential hydration of the caseins.

Keywords: *NMR, ^{17}O, ^{13}C; water binding; sucrose; lactose; bovine casein; caprine casein*

INTRODUCTION

Interactions among food components in food systems have the opportunity to be extensive and may be of extreme importance to the general stability of the food product. The interactions between nonionic solutes and ionic polymers, such as sugar and proteins, have been investigated by several researchers (San Jose et al., 1977; Minson et al., 1981; Lonergan et al., 1981; Arakawa and Timasheff, 1982; Chinachoti and Steinberg, 1988). Improvement in the stability of bovine casein during frozen storage in the presence of sugars such as raffinose, glucose, sucrose, and xylose was shown by Minson et al. (1981) and Lonergan et al. (1981). It was theorized that cryoprotective efficacy was strongly related to a compound's ability to depress the freezing point and to offer many hydrogen bonding sites for interaction with the caseins. Stabilization of proteins by sugars in aqueous, nonfrozen systems, however, appears to be associated with the thermodynamic concept of preferential interactions: a deficiency of the stabilizing sugar (relative to the bulk solution) occurs in the immediate vicinity of the protein (Arakawa and Timasheff, 1982); it was demonstrated that protein is preferentially hydrated. That is, sugars are preferentially excluded from contact with the surface of the protein.

Although the concept of preferential hydration is well understood, the mechanism by which sugar induces the increased stability of, for example, the caseins, on a molecular basis has yet not been elucidated due to the difficulties in determining their molecular conformations. The development of ^{13}C NMR and ^{17}O NMR techniques has shown much promise for the determination of protein conformation, protein−solvent interactions, and protein−protein interactions. Bovine casein micelles and submicelles have been studied by high-resolution ^{13}C NMR, and it has been possible to obtain accurate spectra and identify the carbon peaks (Kakalis et al., 1990). ^{17}O NMR relaxation has also been successfully applied to the study of the extent of hydration of bovine and caprine caseins under experimental conditions such as ionic strength, temperature, and pH (Mora-Gutierrez et al., 1995, 1996a,b). In addition, caprine caseins, in contrast to bovine caseins, vary considerably in the types of casein present; some are rich in α_{s1}-casein, whereas some are poor (Mora-Gutierrez et al., 1996a,b). Previous studies showed major differences in hydration in sucrose among a caprine casein "high" in α_{s1}-casein, a "normal" casein, and a bovine casein (Mora-Gutierrez et al., 1997). NMR techniques have yet to be applied in the investigation of the conformational changes of bovine and caprine caseins which may accompany changes in hydration in the presence of sugars. The objective of this work is to study this sugar−casein interaction and its effect on hydration and protein conformation by use of ^{17}O and ^{13}C NMR techniques and correlate these data with casein type.

MATERIALS AND METHODS

Materials. The following reagents were used: (1) sucrose (MW = 342.30), reagent grade (Sigma Chemical Co.); (2) α-lactose monohydrate (MW = 360.30) (Sigma Chemical Co.), the content of the β-isomer being ∼2%; and (3) deuterium oxide (99.8 atom% D) (Sigma Chemical Co.). Other reagents were of reagent grade.

Sample Preparation. Caseins were obtained from the milk of a Jersey cow and an Anglo-Nubian goat. The Nubian goat

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Table 1. Comparison of the Percentage of Casein Distribution of Bovine and Caprine Caseins by Densitometry

casein type	bovine casein	caprine casein high in α_{s1}-casein[a]
α_{s2}-casein	12.1	9.2
α_{s1}-casein	39.5	25.1
β-casein	37.2	51.6
κ-casein	11.2	13.8

[a] This casein contained one of the highest contents of α_{s1}-casein component found by Mora-Gutierrez et al. (1991).

milk casein was selected as yielding high levels of the α_{s1}-casein component as determined by reversed-phase high-performance liquid chromatography (RP-HPLC) (Mora-Gutierrez et al., 1991). Caseins were isolated from 2 L of fresh, uncooled milk to which phenylmethanesulfonyl fluoride (PMSF; 0.1 g/L) was added inmediately to retard proteolysis. The milk was centrifuged at 4000*g* for 10 min at room temperature to remove the cream fraction. Skimmed milk (500 mL) was diluted with an equal volume of distilled water and warmed to 37 °C. Casein was precipitated by careful addition of 1 N HCl to pH 4.6. The precipitate was homogenized with a Biospec homogenizer at low speed and dissolved by addition of NaOH to yield a solution of pH 7.0. The casein was reprecipitated, washed, and then resuspended. The sodium caseinate was subsequently cooled to 4 °C and centrifuged at 100000*g* for 30 min to remove residual fat. Finally, the casein suspensions were dialyzed exhaustively versus cold deionized water at 4 °C for 72 h and then lyophilized. The integrity of the samples was confirmed by polyacrylamide gel electrophoresis in sodium dodecyl sulfate (PAGE−SDS), and densitometry was used to assess the relative concentrations of casein components (Basch et al., 1989). The compositions of the bovine and caprine caseins used in this study are given in Table 1. The pD of the protein solutions was 7.20. 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 solution in D_2O with the electrode calibrated in standard H_2O buffers. The casein solutions were prepared by stirring the appropiate amount of 99.8% deuterium oxide (D_2O) with casein and sugars. The samples were well mixed over a period of 3 h at 4 °C to reach maximum solubility (Mora-Gutierrez et al., 1993). Samples were allowed to reach room temperature prior to the NMR measurements.

NMR Measurements. Proton-decoupled, natural abundance ^{13}C (50.3 MHz) and ^{17}O (27.1 MHz) NMR measurements were carried out with a multinuclear Varian XL-200 spectrometer (Varian Associates, Palo Alto, CA) operating at 4.7 T. Fourier transforms were carried out on line with a Varian 4000 series data system computer, equipped with Pascal software (v. 6.3).

The number of scans required for a good signal-to-noise ratio (>100:1) in the ^{17}O NMR spectra of sugar−casein samples was ~1000. Line widths were measured at half-height of each ^{17}O NMR spectrum (Figure 1). 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 (ΔR_2, s^{-1}) were then calculated from the line widths by the standard formula:

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

About 4 mL of well-dispersed and throughly mixed protein in D_2O solutions (pD 7.20) was run in 10-mm high-resolution NMR tubes (Wilmad, Buena, NJ). In all experiments two independent series of NMR measurements were conducted at 21 ± 1 °C. The ^{17}O NMR 90° pulse width for D_2O was 19 μs, the recycle time was 0.5 s, and 1024 scans were sufficient for a signal-to-noise ratio of 200:1 (with 5 Hz exponential line broadening applied). The spectral width was 5 kHz, the acquisition time was 0.5 s, and an 8K point time domain array was used for storing the data with adequate resolution.

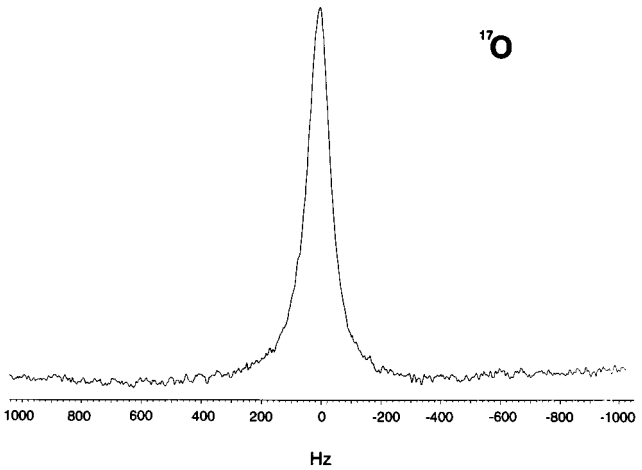

Figure 1. ^{17}O NMR spectrum of D_2O in a 2.26% (w/v) bovine casein solution with 100 mM sucrose at pD 7.20 and 21 ± 1 °C.

For the ^{13}C NMR measurements the pulse width was 7.7 μs (60°) and a recycle time of 1.2 s was used. The spectral width was 11.25 kHz, the acquisition time was 0.7 s, and a 15K point time domain array was used for storing the data. The number of scans required for adequate signal-to-noise ratio was 24000 for sugar−casein mixtures and 1000 for sucrose and lactose. A concentration of ~240 mg/mL (6.0% w/v) protein solution that contained sucrose or lactose was used for the ^{13}C NMR measurements. Solutions were kept overnight at 4 °C with continuous stirring until caseins completely dissolved. The solutions were transferred to 10-mm high-resolution NMR tubes.

For all ^{13}C NMR experiments, sodium 2,2-dimethyl-2-silapentane-5-sulfonate (DSS) was prepared with 100% deuterium oxide and used as the external standard (Perlin and Casu, 1982).

Longitudinal ^{13}C NMR relaxation rates were determined with the inversion recovery method (Vold et al., 1968) using the 180°−τ−90° pulse sequence. The interpulse delay τ was varied from 0.01 to 25 s (seven data points), and the preacquisition delay was set at 35 s. The 90° pulse width was 11.5 μs, the 180° pulse width was 23 μs, and 200 scans were accumulated for each τ value.

The ^{13}C T_1 values were obtained from a nonlinear three-parameter, least-squares fitting of an exponential curve to the experimental points (peak heights) to account for slight pulse width missettings and/or insufficiently long recycle delays (Canet et al., 1975). Proton decoupling was achieved by a bilevel broadband decoupling scheme based on a sequence proposed by Waugh (1982). The decoupler was centered 4.8 ppm downfield from the ^1H resonance of DSS, generating an effective decoupling field of 6.6 kHz. The power output was 9 W during acquisition and 4 W at other times.

Microcomputer Analysis of Experimental Data. The ^{17}O NMR relaxation data were fitted with a Systat (SYSTAT, Inc., Evanston, IL) nonlinear regression program that utilizes a Quasi-Newton algorithm (Systat v. 5.1) 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. The standard deviation, which is the average deviation of the calculated curve from the data points, was calculated as follows:

$$SD = \{[(R_{2calcd} - R_{2obsd})^2]/(\text{no. of data points} - \text{no. of fitting parameters})\}^{1/2} \qquad (2)$$

In eq 2 R_{2obsd} and R_{2calcd} are the observed and calculated transverse relaxation rates, respectively. SD values were normalized to be within at least 5% error of the fit for all the data.

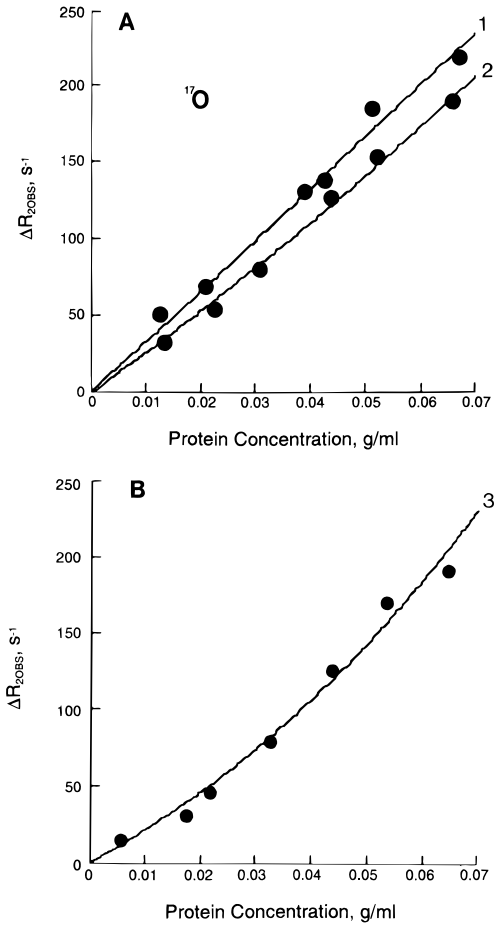

Figure 2. Dependence of the ^{17}O NMR transverse relaxation rates, ΔR_2 (s^{-1}), on protein concentration (g/mL) for bovine casein in the presence of sucrose at pD 7.20 and 21 ± 1 °C: (A) 300 mM sucrose (1) and 200 mM sucrose (2); (B) 100 mM sucrose (3). Data were fitted by eq 6. Results are in Table 2.

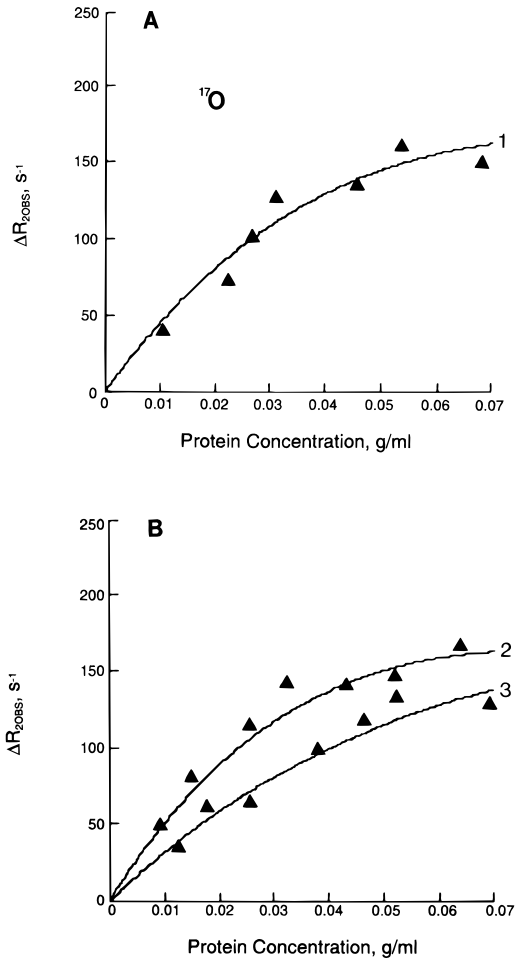

Figure 3. Dependence of ^{17}O NMR transverse relaxation rates, ΔR_2 (s^{-1}), on protein concentration (g/mL) for caprine casein high in α_{s1}-casein in the presence of sucrose at pD 7.20 and 21 ± 1 °C: (A) 300 mM sucrose (1); (B) 200 mM sucrose (2) and 100 mM sucrose (3). Data were fitted by eq 6. Results are in Table 2.

THEORY AND BACKGROUND: ^{17}O NMR RELAXATION

^{17}O NMR transverse relaxation data (R_2) for bovine and caprine casein high in α_{s1}-casein in the presence of sugars are presented in Figures 2−4. To interpret such R_2 NMR relaxation data, we have employed a simple two-state model with fast exchange (Zimmerman and Brittin, 1957) as modified by Pessen and Kumosinski (1985). With increasing concentration of a solute in water, the amount of "free" water decreases, whereas the relative amount of "bound" water increases. If no additional contributions to relaxation were present, a linear relationship between the observed relaxation rate (R_{obsd}) and solute concentration is expected

$$R_{1,2obsd} = P_b R_{1,2b} + P_f R_{1,2f} \quad (3)$$

where P_b and P_f are the percent "bound" and percent "free" water in the system and R_b and R_f are, respectively, the relaxation rates of "bound" and "free" water. In general, this predicted relationship holds for dilute solutions (Derbyshire, 1982). However, departures from linearity are often observed at high concentrations. Three mechanisms to account for this nonlinearity are proposed. Derbyshire (1982) invoked two different mechanisms, a change in the hydration of the macromolecule or a change in the relaxation rate of the "bound" water. The third mechanism, presented by Pessen and Kumosinski (1985), attributes the nonlinearity to charge

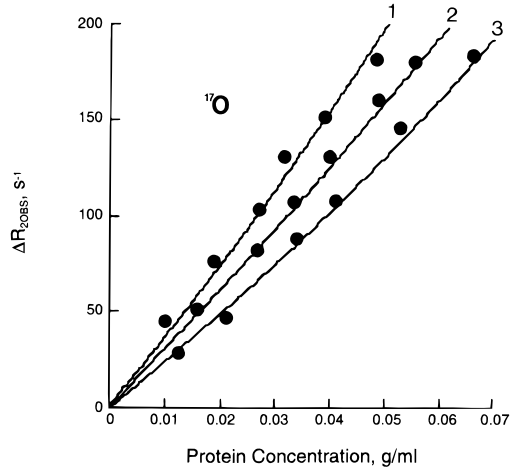

Figure 4. Dependence of ^{17}O NMR transverse relaxation rates, ΔR_2 (s^{-1}), on protein concentration (g/mL) for bovine casein in the presence of lactose at pD 7.20 and 21 ± 1 °C: 300 mM lactose (1); 200 mM lactose (2); 100 mM lactose (3). Data were fitted by eq 6. Results are in Table 2.

repulsion or charge fluctuations as predicted by the Kirkwood−Shumaker (Kirkwood and Shumaker, 1952) theory. The major application of the Pessen and Kumosinski (1985) work to that presented here is the use of activities in place of concentrations when one is

dealing with systems strongly deviating from ideality. The activity of a protein (a_p) in solution is related to its concentration by the activity coefficient γ (eq 4)

$$a_p = \gamma_p C_p \quad (4)$$

where C_p is the protein concentration. The activity coefficient can be obtained from the virial expansion of osmotic pressure as a function of protein concentration

$$\mathrm{d} \ln \gamma / \mathrm{d}C_p = 2B_0 + 3B_2 C_p + ... \quad (5)$$

where the B parameters are the virial coefficients. The virial coefficients are a measure of the various molecular interactions (Tanford, 1963; Pessen and Kumosinski, 1985).

When this virial expansion is applied in conjunction with the two-state model with fast exchange, an equation results for single species proteins

$$R_{2obsd} - R_{2F} = n_H(R_{2B} - R_{2F})C_p \exp[2B_0 C_p + 2B_{0.5}{C_p}^{0.5} + 0.667B_{1.5}{C_p}^{1.5} + 1.5B_2{C_p}^2 + ...] \quad (6)$$

where R_{2obsd} is the measured transverse relaxation rate corrected for inhomogeinity broadening, the subscripts "B" and "F" stand for "bound" and "free" water, n_H is the hydration number (i.e., the average number of water molecules "bound" per molecule of dry protein), and C_p is the varying protein concentration. The B_0 virial coefficient reflects the repulsive or attractive forces arising from the net protein charge Z, the protein excluded volume, and a preferential interaction term

$$2B_0 = Z^2/(4m_s M_p) + \bar{v}_p/1000 - (\partial g_s/\partial g_p)^2 \times 1/m_s \quad (7)$$

where m_s is the sugar molarity, M_p is the average monomer molecular weight of casein, $\bar{v}_p$ is the average partial specific volume of the caseins, and $(\partial g_s/\partial g_p)^2$ is the preferential binding term (grams of preferentially "bound" sugar per gram of protein); this preferential interaction term must be included to take into account the preferential interaction of sugars and water at the protein interface (Arakawa and Timasheff, 1982; Kumosinski et al., 1987). The B_2 virial coefficient represents attractive forces arising from fluctuating multipoles. For isoionic protein solutions (i.e., in the absence of any ions), the $B_{0.5}$ and $B_{1.5}$ terms must be included in eq 6 to account for attractive effects due to charge fluctuations (Kirkwood and Shumaker, 1952; Timasheff 1982; Pessen and Kumosinski, 1985). Essentially, the exponential term of eq 6 represents the activity coefficient; therefore, determination of these virial coefficients will allow for quantitation of the protein−protein interactions or the protein activity.

RESULTS AND DISCUSSION

Determination of Virial Coefficients and Apparent Hydration. Casein hydration and protein activity were determined by nonlinear regression analysis of the ^{17}O NMR relaxation data using eq 6. The plots shown in Figures 2−4 indicate that the experimental data are fitted quite well with the calculated data of ^{17}O NMR relaxation. In the protein concentration range from 0.1 to 7.0% (w/v) no other virial coefficient other than $2B_0$ was necessary to fit the oxygen-17 NMR data as judged by no significant improvement in SD. The deviations from linearity are caused by protein activity effects (i.e., changes in B_0). The results shown in Table 2 indicate that the stabilizing effects of the two sugars are similar in that they both caused a decrease in B_0, which denotes a decrease in charge−charge repulsive interactions at high concentrations. This general decrease in repulsive interactions increases the thermodynamic stability of the system. However, the extent of sugar stabilization is different (B_0 values; Table 2), reflecting the importance of the molecular configuration of the sugars and the combination of casein types in these milk proteins. As shown in Table 2, the extent of sucrose stabilization is more accentuated with the caprine casein high in α_{s1}-casein, which contains a greater content of the hydrophobic β-casein than bovine casein (Table 1). Concentrated lactose solutions (300 mM) of caprine casein high in α_{s1}-casein also exhibited a significant stabilizing effect ($B_0 = -3.1$ mL/g).

Table 2. Calculated Hydration Products $n_H\Delta R$[a] and Virial Coefficients[b] from Nonlinear Regression Analysis of Oxygen-17 NMR Transverse Relaxation Data for Bovine and Caprine Caseins in Deuterated Sucrose and Lactose Solutions at pD 7.20 and 21 ± 1 °C Using Equation 6

	sugar type and concn (mM)			
casein	type	concn	$n_H\Delta R$	B_0
bovine	none	0	1668.3 ± 26.4	3.9 ± 0.3
	sucrose	100	1986.4 ± 19.2	3.7 ± 0.2
	sucrose	200	2545.5 ± 23.7	1.2 ± 0.2
	sucrose	300	3231.9 ± 25.4	0.4 ± 0.1
caprine high in α_{s1}-casein	none	0	842.8 ± 16.7	2.6 ± 0.3
	sucrose	100	3437.0 ± 33.8	−4.0 ± 0.2
	sucrose	200	5178.4 ± 38.3	−6.5 ± 0.1
	sucrose	300	5061.1 ± 36.7	−5.6 + 0.1
bovine	none	0	1668.3 ± 26.4	3.9 ± 0.3
	lactose	100	2600.5 ± 30.9	0.7 ± 0.2
	lactose	200	3101.6 ± 26.8	0.5 ± 0.2
	lactose	300	3582.7 ± 26.4	1.1 ± 0.1
caprine high in α_{s1}-casein	none	0	842 ± 16.7	2.6 ± 0.3
	lactose	100	1406.5 ± 27.9	1.5 ± 0.3
	lactose	200	1934.7 ± 24.7	1.2 ± 0.2
	lactose	300	3583.6 ± 31.7	−3.1 ± 0.2

[a] ln mL g^{-1} s^{-1}. The protein concentration was in g of protein/mL of solvent. [b] In mL/g.

In the case of bovine casein, maximum stability was obtained with 100−200 mM lactose ($B_0 = 0.7$ and 0.5 mL/g, respectively). Nevertheless, lactose at a level of 300 mM led to a slight decrease in stability ($B_0 = 1.1$ mL/g). These results, with sodium caseinate, agree with those obtained with lactose in frozen milk (Rose, 1956). However, it is interesting to note that in the milk−lactose system, interactions with milk components (i.e., casein, salt) were dependent on the content of these latter two milk components (Rose, 1956). That is, high levels of casein and salt made suspensions in higher lactose concentrations more stable.

The magnitude of the hydration product ($n_H\Delta R$ of Table 2) may be affected by (1) the apparent number of water molecules (P_b), their relaxation rate (R_b), or both (see eq 3); (2) increases in solvent viscosity due to increases in solute (sugar) concentration; (3) increases in protein−protein association reactions; or (4) direct formation of sugar−protein complexes (aggregates). Although some researchers (Tait et al., 1972; Sugget et al., 1976) have observed line broadening in the presence of dissolved mono- and disaccharides, the increase in the average hydration parameter, which includes the relaxation rate for "bound" water, of bovine and caprine

caseins in sucrose and lactose solutions (Table 2) may not be solely explained as arising from viscosity effects. The effect of sugars on the stability of proteins in water has more recently been interpreted in terms of changes in protein conformation, state of aggregation of the proteins, and changes in the interactions between protein and solvent components (Lee and Timasheff, 1981; Arakawa and Timasheff, 1982). The overall decreases in B_0 seen in this work indicate a general decrease in electrostatic repulsions as the sugar concentrations are increased. The best interpretation of our data would be that sugars cause protein stabilization most likely through preferential hydration and not by direct binding to the protein. That is, the water layer around the protein is enriched in water relative to the sugar−water solvent, and as the concentration of the sugar increases the preferential hydration increases as well.

The ^{17}O NMR technique measures protein in solution based on changes in molecular mobility of the water associated with the protein or the sugar−protein system. Because it is the water being probed and not the protein or the sugar−protein system directly, the method is an indirect measurement of the sugar−protein interaction. The preferential parameter is derived as either plus or minus from eq 7 (Mora-Gutierrez et al., 1997). To characterize the sugar−protein interaction further, we now resort to a response of the ^{13}C NMR spectrum of the sugar to these environmental conditions.

^{13}C NMR. The chemical shift of a specific carbon atom depends on the degree of shielding by its surrounding electrons. If any interaction between sugar and protein occurs, the environment near those protein carbon atoms would be altered, and in turn its chemical shift could also be altered. Therefore, chemical shift differences in the ^{13}C NMR spectra of the sugar−protein system may be used to monitor possible changes in the carbon chain of the protein as a function of added sugar.

^{13}C NMR spectra were obtained for all samples. Figure 5 shows a comparison among spectra of pure sucrose (A), a sucrose−bovine casein mixture (B), and pure bovine casein (C). The carbon peaks for sucrose and lactose were located between 60 and 120 ppm (Figures 5A and 6A, respectively). Although bovine casein (Figure 5C) showed a small number of peaks within this range, the bovine casein and the sucrose spectra did not heavily overlap (Figure 5B). Assignment of carbon peaks was according to results given by Kakalis et al. (1990) for bovine caseins. Assignments of certain major ^{13}C carbon resonances are in Table 3. The differences in chemical shifts of any carbon atom in the bovine casein and the caprine high α_{s1}-casein molecule over all sucrose concentrations were <0.2 ppm. On the basis of lack of chemical shift differences in the ^{13}C NMR spectra, no significant perturbations of the carbon atoms of these two casein complexes appear to occur as a result of added sucrose. Thus, no sugar binding is detected by this method.

Dynamics of Sucrose Carbons. ^{13}C NMR studies were conducted for sucrose in the presence of protein. The nearly identical T_1 values for the protonated carbon atoms of sucrose in a caprine casein high in α_{s1}-casein at constant protein concentration (6.39% w/v) in D_2O as a function of sucrose concentration (Table 4) suggest that the dynamic states of the carbon atoms in sucrose are not affected by sugar viscosity. Such uniformity of T_1 values suggest only isotropic tumbling of the sugar (Bock and Lemieux, 1982) in the presence of the proteins. This strongly suggests that the sucrose does not bind to the protein molecules as T_1 values are not altered by the presence of protein. This means that preferential hydration occurs as suggested earlier.

Estimation of the Degree of Preferential Hydration by ^{17}O NMR Relaxation. Although the relaxation increment (ΔR_2) is influenced by viscosity, the ^{13}C NMR data suggest it does not make a major contribution here. It is of interest to estimate the magnitude of the preferential interaction term ($\partial g_s/\partial g_p$) from eq 7. The problem with this equation is that the preferential term occurs as a square so that its solution is not exact and yields a ± sign. However, as no direct protein−sugar interactions were detected (above) and as the overwhelming literature evidence argues, the preferential interaction term here (Arakawa and Timasheff, 1982) is considered negative, describing preferential hydration (a plus sign would indicate preferential binding of sugar). The calculated values for the preferential interaction terms are given in Table 5. The values calculated from the oxygen-17 NMR data for the preferential hydration of caseins by sugars are in excellent agreement with those calculated by densimetry for globular proteins (Arakawa and Timasheff, 1982). Additionally, the increase in magnitude of the term with increasing sugar follows the trends previously reported for globular proteins (Arakawa and Timasheff, 1982) and for tubulin (Na and Timasheff, 1981). Thus, the oxygen-17 NMR estimates of B_0 and $\partial g_s/\partial g_p$ appear to yield good approximations of the terms even though the effect of viscosity on τ_c is not taken into account. Bearing this caution in mind, the average number in Table 5 is 0.0590 g of sugar excluded/g of protein. This translates to 43 mol of sugar excluded/250000 g of protein, which is the approximate molecular weight of the average submicelle.

Table 2 shows that the average hydration product, which includes the relaxation rate for "bound" water and the apparent number of "isotropically bound" water molecules, increases when sugar is added. It is seen that the effect of sucrose and lactose on the hydration product $n_H\Delta R$ (Table 2) strongly depends on both the sugar and the casein.

By making assumptions regarding the nature of the "bound" water, R_{2B} can be calculated and used to dissect the hydration product to yield an apparent "isotropically bound" hydration number (Mora-Gutierrez et al., 1995). The calculated hydration from oxygen-17 NMR data given in Table 6 shows the effect of increasing sugar concentrations. The hydration of both of the caseins increases with increasing sugar concentration. These values represent estimates of "bound" water because the water sensed by the oxygen-17 nucleus in the sugar−casein systems is exchangeable water "trapped" within the casein complexes and not water merely influenced by the caseins. This conclusion is borne out by the ^{17}O NMR experiments of Denisov and Halle (1995), who showed that for bovine pancreatic trypsin inhibitor (BPTI) the classes of water molecules included 4 internal (trapped) water molecules and 250 surface water molecules, but the 4 internal water molecules contribute disproportionately to the relaxation. Although we also observe a weighted average for the relaxation and are studying a more complex protein system in our work, internal or "trapped" water undoubtedly dominates our experiments as well. This dominance is true for the

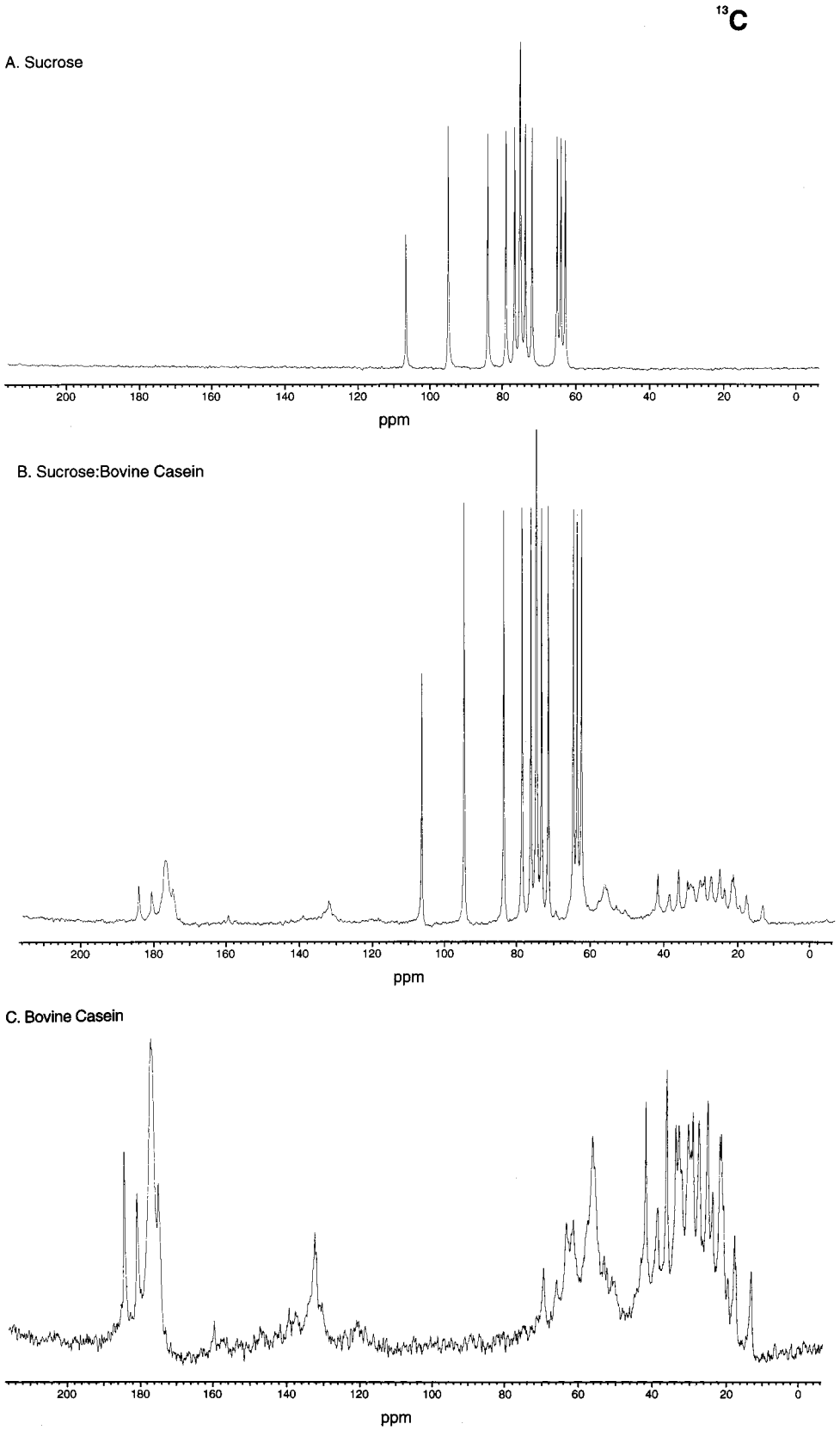

Figure 5. ^{13}C NMR spectra of (A) sucrose, (B) sucrose−bovine casein [100 mM sucrose/6.62% (w/v) bovine casein], and (C) bovine casein in D_2O at pD 7.20 and 21 ± 1 °C. Proton broad-band decoupling and other experimental conditions were as specified under Materials and Methods.

casein aggregates, which provide many internal cavities and surface pockets for water. Therefore, our relaxation experiments report changes undergone by this "trapped" water and not water transiently "bound" to surface charges.

Nevertheless, in the presence of sugars the average hydration value of Table 6 is 0.0057 g of water/g of protein; in the absence of sugar this value is 0.0030. This translates to ∼40 more water molecules. Thus, increased "bound" water represents a small fraction of the

Table 3. Observed Carbon-13 NMR Chemical Shifts[a] and Line Widths of Certain Bovine and Caprine Casein Resonances in Deuterated Sucrose Solutions at pD 7.20 and 21 ± 1 °C

		casein			
		chemical shift (ppm)		line width (Hz)	
sucrose (mM)	resonance	bovine	caprine high in α_{s1}-casein	bovine	caprine high in α_{s1}-casein
0	Phe ϵCH, Tyr δCH	131.90	131.90	104.67	51.37
	Gln δCO	180.38	180.38	46.69	42.33
	Glu δCO	183.82	183.93	29.79	21.54
100	Phe ϵCH, Tyr δCH	131.90	–[b]	85.85	–
	Phe δCH, Tyr δCH	–	131.46	–	55.58
	Gln δCO	180.35	180.38	45.54	42.52
	Glu δCO	183.85	183.90	26.89	22.33
200	Phe ϵCH, Tyr δCH	–	–	–	–
	Phe δCH, Tyr δCH	131.46	131.46	102.63	49.91
	Gln δCO	180.30	180.32	38.10	42.17
	Glu δCO	183.79	183.87	23.83	22.57
300	Phe ϵCH, Tyr δCH	131.85	–	143.15	–
	Phe δCH, Tyr δCH	–	131.46	–	48.61
	Gln δCO	180.32	180.30	43.76	36.89
	Glu δCO	183.76	183.85	28.62	20.86

[a] Relative to DSS as an external reference. [b] Broad/weak signal.

Table 4. ^{13}C Spin–Lattice Relaxation Times (in Seconds) for the Protonated Carbon Atoms of Sucrose in a Caprine Casein High in α_{s1}-Casein (6.39% w/v) Solution in D_2O at 21 °C[a]

chemical shift (ppm)	sucrose (mM)				
	50	100	200	300	av ± SD
106.35[b]					
94.83	0.42	0.42	0.39	0.37	0.40 ± 0.02
84.02	0.38	0.40	0.37	0.35	0.38 ± 0.02
79.05	0.37	0.41	0.41	0.37	0.38 ± 0.02
76.65	0.48	0.45	0.40	0.38	0.43 ± 0.04
75.20	0.44	0.48	0.42	0.40	0.43 ± 0.03
75.07	0.45	0.46	0.42	0.39	0.44 ± 0.02
73.73	0.47	0.42	0.44	0.38	0.43 ± 0.03
71.87	0.45	0.44	0.41	0.39	0.42 ± 0.02
65.05	0.29	0.28	0.28	0.26	0.28 ± 0.01
64.01	0.27	0.24	0.24	0.23	0.25 ± 0.02
62.78	0.30	0.23	0.24	0.23	0.25 ± 0.03

[a] The error in the ^{13}C T_1 measurements is of the order of 4%. [b] This represents the C_2 residue of the sucrose moeity; it is not protonated.

water molecules associated with highly porous caseins. It can be shown that the preferential hydration caused by exclusion of 43 sugar molecules may be more significant. Using the equations of Arakawa and Timasheff (1982), it can be calculated that in 200 mM lactose a value of $\partial g_s/\partial g_p$ of 0.0522 as given in Table 5 for bovine casein leads to a value of 0.7250 for $\partial g_w/\partial g_p$. This represents an enormous increase in solvation of the casein. The net result is quite dramatic in that these forces enhance casein stabilization as judged by favorable changes in B_0 and in the curvature of the raw data shown in Figures 2–4.

Interpretation of the results in terms of increased state of aggregation of the caseins is feasible. However, the porous nature of the casein submicelles might yield the reverse effect. The presence of the sugars and resultant hydration could disaggregate the highly rugose submicelles and lead to smaller more compact structures with greater stability and perhaps lower surface area. Alternatively, the submicelles may merely become more compact in response to the presence of the sugars. No experimental evidence is apparently available on the effects of sugars on the size and/or shape of submicellar caseins. However, Dewan et al. (1973) noted that for colloidal calcium caseinates (micelles) 10% sucrose decreased the voluminosity of the micelles by 20%. In addition, Mozersky et al. (1991) were also able to detect a significant decrease in MW of reformed micellar protein aggregates of bovine whole caseins in the presence of sucrose and lactose by sedimentation field flow fractionation. In analogy with globular proteins, both sugars are expected to favor association of molecular dispersed caseins (Lee and Timasheff, 1981; Arakawa and Timasheff, 1982). In this context, the values of the B_0 virial coefficients in the presence of sugars are in magnitude smaller than the values estimated for bovine and caprine caseins in the absence of these two sugars (Table 2). This indicates that it is likely that sucrose and lactose stabilize bovine and caprine caseins by causing preferential hydration and thus decreasing electrostatic interactions between oppositely charged groups of these milk proteins. Thus, the mechanism of bulk surface tension changes is at work in these studies, as suggested by Arakawa and Timasheff (1982). Changes in preferential hydration relate to large changes in the macromolecule. Because a large and positive B_0 term may be interpreted as an indication of increased charge repulsions due to protein aggregation (Pessen and Kumosinski, 1985), it is demonstrated by this experiment that the reduction in B_0 predicts increases in the stability of the caprine caseins with sugar concentration (Table 2). For our systems it is obvious that the functionality of the casein aggregates formed by the attractive interaction depends on the types of the caseins as well as the concentration of the sugar present as previously reported for reformed micelles (Mozersky et al., 1991).

Apparently the preferential effect in sugar–casein mixtures is more predominant among caprine caseins, and this ability seems to be related to their distinct compositional characteristics (Grosclaude et al., 1987; Mora-Gutierrez et al., 1991) and resulting influences on physicochemical properties (Mora-Gutierrez et al., 1993, 1995, 1996a,b). In this context, it is possible that the high content of β-casein of the caprine casein preparation (Table 1) favors the formation of highly hydrated casein aggregates. The β-casein molecule itself possesses a large hydrophobicity (Farrell, 1988) and a high voluminosity, which has been ascribed to water entrapped in the spatial structure (Le Meste et al., 1990; Kumosinski et al., 1993). The above results clearly indicate that the hydration product $n_H\Delta R$, the preferential binding term $\partial g_s/\partial g_p$, and protein hydration (Tables 2, 5, and 6, respectively) are strong functions of the chemical nature of the protein composition in both the sucrose and lactose systems.

Thus, the influence of a given solute on the stability of a protein derives from a balance between the destabilizing effects of solute binding to a protein and the stabilization imparted when the solute is excluded from the protein (Timasheff, 1982). The relative contributions of either force depend on the surface properties of the protein as well as the concentration of solute present. Results of the preferential interaction term ($\partial g_s/\partial g_p$; Table 5) seem to indicate that sucrose and lactose "preferentially" interact with these two milk proteins. This sugar–casein interaction is likely to foster preferential hydration (Tables 2 and 6). In addition, the observed solvent interactions are related to the chemical

Table 5. Calculated Preferential Interaction Term ($\partial g_s/\partial g_p$) for Bovine and Caprine Caseins in Deuterated Solutions of Sucrose and Lactose at pD 7.20 and 21 ± 1 °C Using Equation 7

casein	sucrose (mM)	$-\partial g_s/\partial g_p$[a]	$-\partial g_s/\partial g_p$[b]	lactose (mM)	$-\partial g_s/\partial g_p$[a]	$-\partial g_s/\partial g_p$[b]
bovine	100	0.0460		100	0.0521	
	200	0.0495		200	0.0522	
	300	0.0526		300	0.0484	
caprine high in α_{s1}-casein	100	0.0605	0.0492	100	0.0505	0.0363
	200	0.0744	0.0655	200	0.0495	0.0348
	300	0.0798	0.0716	300	0.0698	0.0602

[a] g of sugar/g of protein. Calculated with B_0 values from Table 2; M_p and Z were chosen as 23300 Da and −16.1 esu, respectively (Eigel, 1984), and a $\bar{v}_p$ of 0.736 (Kumosinski et al., 1987) was taken for an average partial specific volume of the bovine caseins. [b] g of sugar/g of protein. Calculated with B_0 values from Table 2, as above, except Z was chosen as −12.0 for the caprine casein on the basis of its composition (Table 1).

Table 6. Hydration Estimates of Bovine and Caprine Caseins[a]

casein	sucrose (mM)	hydration,[b] g of water/g of protein	lactose (mM)	hydration,[b] g of water/g of protein
bovine	0	0.00452	0	0.00452
	100	0.00428	100	0.00687
	200	0.00498	200	0.00813
	300	0.00605	300	0.00830
caprine high in α_{s1}-casein	0	0.00097	0	0.00097
	100	0.00527	100	0.00281
	200	0.00528	200	0.00586
	300	0.00673	300	0.00772

[a] From oxygen-17 NMR data at 21 ± 1 °C and at pD 7.2, according to a two-state, isotropic model (Mora-Gutierrez et al., 1995). [b] Assuming $\tau_c = 56$ ns for bovine casein submicelles (Kakalis et al., 1990).

nature of the protein surface in both the sucrose and lactose systems.

Dynamics of Casein Chains as a Result of Preferential Hydration. To assess the effects of preferential hydration on local motion, two sets of protein peaks were selected for line width measurements. Gln and Glu δCO were chosen to represent exterior hydrophilic residues, whereas Phe ϵCH and δCH and Tyr δCH were chosen to represent interior hydrophobic residues. Experimentally measured line widths are presented in Table 3. In the absence of sugar, the smaller values suggest faster segmental motion for most carbon atoms within the caprine casein high in α_{s1}-casein relative to the bovine caseins. It should be noted that caprine casein high in α_{s1}-casein has a higher content of β- and κ-caseins than bovine casein (Table 1). The β-casein molecule and the κ-casein molecule, particularly its macropeptide tail (Rollema et al., 1988), should be more flexible than the α_{s1}-casein molecules. Thus, the segmental flexibility of the caprine casein high in α_{s1}-casein particles (relative to that for the bovine particles) may be associated with a higher proportion of protein components (β- and κ-caseins) that are more flexible.

It is known that the tryptophanyl group is the most hydrophobic group of proteins followed by the tyrosyl and phenylalanine groups (Tanford, 1970). The small decrease in line width of the carbonyl groups (Gln δCO, Glu δCO) clearly indicates that there is not a significant increase in restriction of motion or in the degree of hydrogen bonding around the carbonyl groups for bovine casein. For sucrose and caprine high in α_{s1}-casein there is also no significant change in the mobility of the carbonyl groups and no significant response of the aromatic line widths in the caseins. The only exception is the 131.85 ppm (Phe ϵCH, Tyr δCH) for bovine casein at 300 mM sucrose; this could be an outlying point. Overall this is evidence that, only very limited changes in hydrophobic interactions occur as a result of preferential interactions induced by sucrose.

^{13}C NMR spectra were also recorded for bovine and caprine casein solutions to which lactose was added to make a total sugar concentration of 100, 200, and 300 mM. The highly detailed ^{13}C NMR spectrum of lactose is shown in Figure 6A. Most resonances for this sugar sample fall within the range from 60 to 110 ppm. Figure 6B shows the ^{13}C spectrum of caprine casein high in α_{s1}-casein to which 200 mM lactose is added. The spectrum of the caprine casein high in α_{s1}-casein, Figure 6C, is not heavily marked by overlap of the carbon resonances of lactose (Figure 6B).

Table 7 compares the chemical shifts and line widths for the bovine casein and the caprine casein high in α_{s1}-casein obtained in the absence of lactose and those obtained for the caseins in the presence of lactose at concentrations of 100, 200, and 300 mM. Again, the chemical shifts were consistent with those of the sucrose−casein samples (Table 3), and the peaks were not significantly shifted as a result of added lactose. The negligible changes in chemical shifts again suggest that the lactose−casein interaction is not direct binding. Here again, the smaller observed values of line widths in caprine casein high in α_{s1}-casein reflect the fact that the motions of the protein side chains are higher than those of bovine casein (Table 7). As noted above, additional β-casein and κ-casein produce no significant differences in the spectra (Figure 6) but may increase the mobility of protein side chains for the caprine casein high in α_{s1}-casein (Table 7).

Lactose, which is the most abundant low molecular weight solute in bovine and caprine milks (~4.5 wt % or 135 mM; Parkash and Jenness, 1968; Swaisgood, 1985), induces few dynamic changes in the bovine casein molecule (Table 7) that differ from those of sucrose. Again, the dynamic changes at carbonyl carbon sites show no responses to the concentration of lactose used for both the bovine and caprine caseins.

Concluding Remarks. The ^{13}C NMR results appear to offer a consistent and widely applicable explanation of observed bovine and caprine casein ^{17}O NMR ΔR_2 relaxation. It may be concluded from this work that sucrose and lactose foster both increased hydration (Table 6) and preferential hydration (Table 5) in bovine casein and caprine casein high in α_{s1}-casein. The line width effects are concluded to be due to changes in hydrophobic regions containing aromatic carbon groups and not the contribution of viscosity or direct sugar binding.

All bovine line widths associated with hydrophobic groups were greater than the caprine line widths, so the dynamics of aromatic carbon groups were clearly

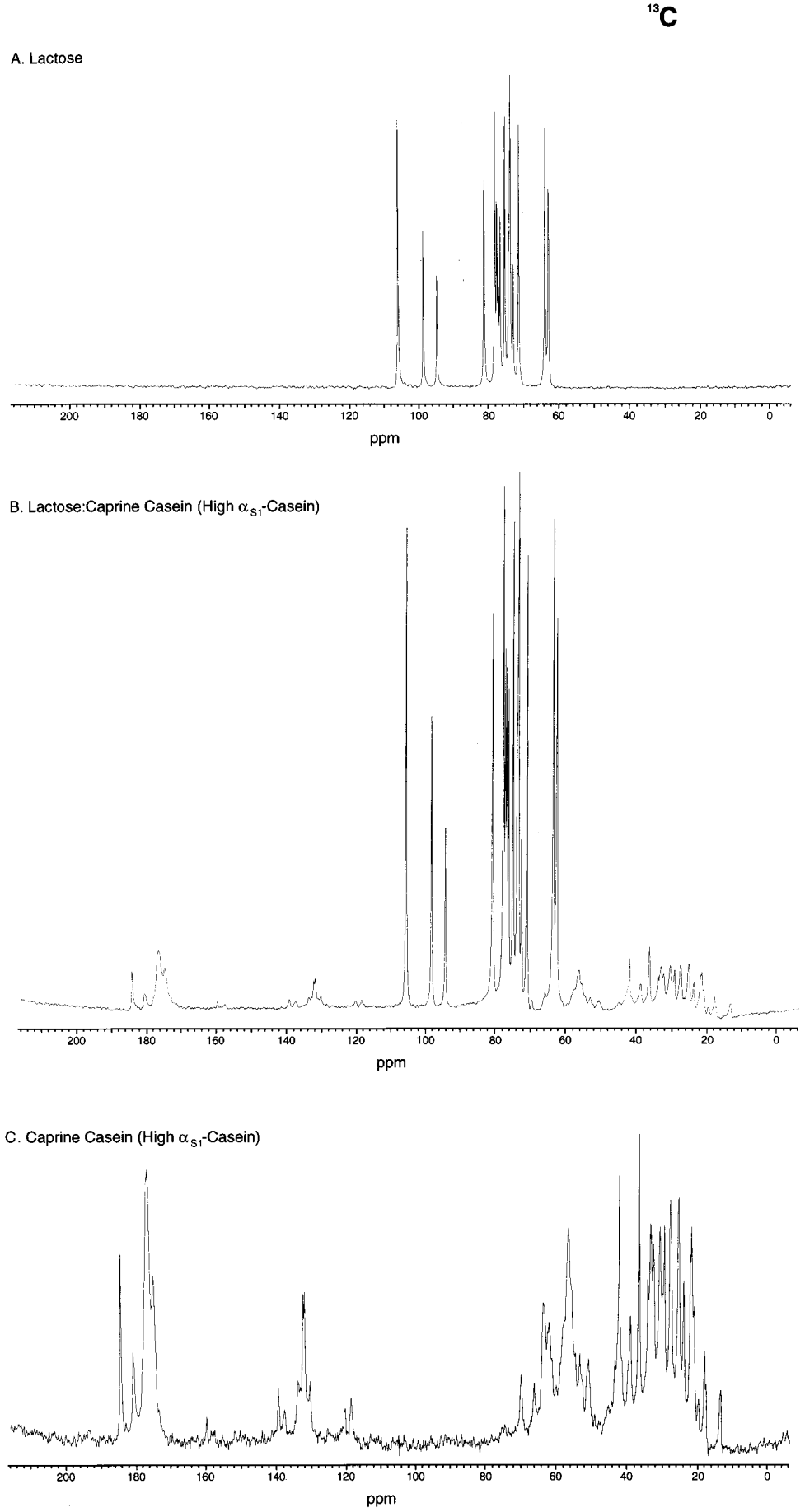

Figure 6. ^{13}C NMR spectra of (A) lactose, (B) lactose−caprine casein high in α_{s1}-casein [200 mM lactose/6.73% (w/v) caprine casein high in α_{s1}-casein], and (C) caprine casein high in α_{s1}-casein in D_2O at pD 7.20 and 21 ± 1 °C. Proton broad-band decoupling and other experimental conditions were as specified under Materials and Methods.

Table 7. Observed Carbon-13 NMR Chemical Shifts[a] and Line Widths of Certain Bovine and Caprine Casein Resonances in Deuterated Lactose Solutions at pD 7.20 and 21 ± 1 °C

		casein			
		chemical shift (ppm)		line width (Hz)	
lactose (mM)	resonance	bovine	caprine high in α_{s1}-casein	bovine	caprine high in α_{s1}-casein
0	Phe ϵCH, Tyr δCH	131.90	131.90	104.67	51.37
	Gln δCO	180.38	180.38	46.69	42.33
	Glu δCO	183.82	183.93	29.79	21.54
100	Phe ϵCH, Tyr δCH	131.93	131.87	84.15	75.70
	Gln δCO	180.30	180.38	35.24	53.26
	Glu δCO	183.76	183.90	22.62	22.95
200	Phe ϵCH, Tyr δCH	131.98	—[b]	102.26	—
	Phe δCH, Tyr δCH	—	131.54	—	47.38
	Gln δCO	180.41	180.41	47.09	40.43
	Glu δCO	183.85	183.93	25.81	21.85
300	Phe ϵCH, Tyr δCH	131.90	131.82	74.44	53.81
	Gln δCO	180.32	180.27	30.10	38.20
	Glu δCO	183.76	183.79	23.32	20.10

[a] Relative to DSS as an external standard. [b] Broad/weak signal.

associated with casein composition (bovine versus caprine) rather than with concentration of the sugar or the kind of sugar.

ABBREVIATIONS USED

NMR, nuclear magnetic resonance; SD, standard deviation; D_2O, deuterium oxide; DSS, sodium 2,2-dimethyl-2-silapentane-5-sulfonate.

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