¹H Nuclear Magnetic Resonance Relaxometry Study of Water State in Milk Protein Mixtures

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¹H NMR signal was used to characterize highly hydrated milk protein dispersions (3−20% dry matter) with various micellar casein concentrations (3−15%), whey protein concentrations (0−3%), lactose concentrations (0−7.5%), $CaCl_2$ concentrations (0−2 mM), and pH (6.2−6.6). The results showed the predominant effect of micellar casein concentration on water state and were consistent with the three-site relaxation model in the absence of lactose. The relaxation rates observed for these dispersions were explained by the free water relaxation rate, the hydration water relaxation rate, and the exchangeable proton relaxation rate. Hydration water was found to be mainly influenced by casein micelle concentration and structure. The variations in hydration with pH were consistent with those observed for classical measurement of voluminosity observed at this range of pH. The effects of lactose and whey protein content are discussed.

KEYWORDS: Casein; lactose; hydration water; NMR relaxation; pH effect

INTRODUCTION

Interactions between water and proteins are usually classified into various categories (*1, 2*). However, the boundaries between these categories are not clearly defined and generally depend on the method of measurement used (*3*). These methods are calorimetry (*4*), sorption isotherms (*5, 6*), NMR (*7−9*), ultracentrifugation (*10, 11*), and osmotic pressure measurements (*12*). Of all these methods, NMR is particularly attractive for the characterization of a heterogeneous system such as milk, as it is both nondestructive and noninvasive. Numerous NMR relaxation studies have been performed on casein−water systems using ¹H, ²H, or ¹⁷O nuclei as probes. Some of them have investigated the water−casein interactions in milk (*8, 13−16*) and in milk powders, and others have dealt with isolated milk components such as sugars (*7*), caseins (*17, 18*), caseinate (*9, 19−24*), and whey proteins (*25*).

¹⁷O NMR relaxation measurements have been used to investigate the effects of NaCl, $CaCl_2$, and sucrose concentrations, temperature, and pH on casein hydration (*22−24, 26, 27*). For example, addition of $CaCl_2$ to a caseinate solution induced an increase in the amount of water "trapped" in the casein structure (from 0.00652 to 0.0165 g of water/g of protein) (*20*). This effect was directly related to the change in the casein structure from submicellar to micellar structure induced by the calcium. Moreover, a higher degree of hydration was observed upon cooling from 30 to 2 °C. The sensitivity of hydration

according to temperature was explained by the effect of the hydrophobic interaction on structure looseness. The water−casein interaction has also been compared in bovine and caprine casein, and a casein monomer composition effect has been demonstrated on the hydration.

Water hydration in casein solutions has also been investigated by ²H and ¹H NMR relaxation measurements, although the relaxation mechanisms involved are different. Contrary to ¹⁷O, ¹H relaxation is very sensitive to the chemical exchange mechanism, and this complicates the interpretation of relaxation time parameters. Nevertheless, similar variations in the water relaxation rate have been observed between ¹⁷O and ¹H relaxation measurements according to the milk pH. When the pH decreases, the structure of the micellar casein changes due to release of the calcium phosphate complex into the soluble phase. The release is maximum at pH 5.3 (*28*) and both the ¹⁷O and ¹H water relaxation rates are minimum (*9*), and at this pH the micellar structure is modified (*28−30*). Moreover, this does not occur with sodium caseinate solution. In this case the dependency of the water relaxation time on pH is negligible compared to variations observed with micellar casein dispersion.

All of these studies have demonstrated the high sensitivity of water relaxation on casein micelle concentration and structure. Most studies have been carried out on a simple casein−water solution or directly on milk reconstituted from milk powder. However, there have been few studies on the effects of other components of milk such as soluble protein and lactose on the relaxation rate.

The aim of this study was to investigate the effects of various concentrations of micellar casein, soluble protein, lactose, and $CaCl_2$ and pH on ¹H NMR relaxation parameters. To quantify

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the effects of components and the interactions between them, an experimental design was carried out.

THEORY

The ^{1}H NMR relaxation of water in highly hydrated protein dispersions (water content >60%) involves different mechanisms. The relaxation mechanisms usually considered to explain water proton relaxation are (1) the chemical exchange between labile protein protons and water protons (*8*), (2) cross-relaxation between bulk water protons and protons of macromolecules (*31*), and (3) exchange of internal water molecules and bulk water (*32*). Venu et al. (*32*) demonstrated that the cross-relaxation mechanism was negligible and that the dominant effects on spin−spin relaxation were proton exchange and water molecule exchange.

Consequently, if the fast diffusive exchange condition between water molecules is confirmed, then a single relaxation rate for water can be measured and the three-site exchange model could be applied (*33, 34*). This model considers three categories of protons: (1) "free" water protons, the mobility of which does not depend on macromolecule motion; (2) hydration water protons, the mobility of which depends on macromolecules motion; and (3) water protons in exchange with exchangeable protein protons. For protein dispersion, this model is generally characterized by a linear relationship between relaxation rate and protein concentration, and the equation usually used to describe relaxation is the following:

$$R_{2obs} = R_{2a} + nC_p(R_{2b} - R_{2a}) + P_c\left(\frac{K_c R_{2c}}{K_c + R_{2c}} - R_{2a}\right) \quad (1)$$

Subscripts a, b, and c refer to bulk water, hydration water, and exchangeable macromolecule protons, respectively. R_{2i} is the spin−spin relaxation rate, P_i the relative population, and K_c the chemical exchange rate. The protein concentration is C_p, and the amount of hydration water expressed in grams of water per gram of protein is n. This relationship has been validated in numerous pure protein dispersions (*8, 34, 35*). For more complex media it was necessary to consider the hydration water fraction from different kinds of molecules and their exchangeable protons.

This model has been used by Hills et al. (*8*) and Mariette et al. (*9*) on reconstituted milks from milk powders. The authors found that for milk it was possible to describe relaxation with eq 1, despite the presence of various molecules. Because of the high hydration potential of micellar casein, they observed that the effect of chemical exchange on water relaxation could be mainly attributed to the soluble protein (whey protein) and lactose. Micellar casein contributes mainly to the water exchange between bulk water and internal water molecules. Consequently, in a pure micellar solution the relaxation observed should be described by the simple two-site fast diffusive exchange model:

$$R_{2obs} = R_{2a} + nC_p(R_{2b} - R_{2a}) \quad (2)$$

Although this model has been considered to be able to describe water proton relaxation in milk, it has not been evaluated for varying casein concentrations, and the effects of other components such as lactose and whey protein have never been investigated.

MATERIALS AND METHODS

Materials. Protein dispersions were prepared with native phosphocaseinate powders (INRA, Rennes, France), whey proteins (Armor Proteines, St. Brice et Cogles, France), CaCl$_2$ (Ultrapur, Merk,

Table 1. Casein Dispersion Composition and Milk Composition for the Experimental Design

	factor				
level	NPC (g/100 g)	WP (g/100 g)	CaCl$_2$ (mM)	pH	lactose (g/100 g)
−1	3	0.0	0	6.2	0
0	9	1.5	1	6.4	3.75
+1	15	0.03	2	6.6	7.5
milk	3	0.03	0	6.4	4.9

Darmstadt, Germany), lactose monohydrate (Merk, Darmstadt, Germany), and lactic acid (Merck).

Sample Preparation. A set of samples containing various native phosphocaseinates (NPC), lactose (Lac), whey protein (WP), and CaCl$_2$ were prepared at different pH values. Sample composition is reported in **Table 1**. According to dispersion composition, NPC was dispersed at 4 °C with a Kenwood mixer (4000 rpm) in an aqueous or lactose solution prepared in distilled water, at an ionic strength of 0.1 M (NaCl). The dispersion was then left at room temperature (20 ± 2 °C) for 1 h and mixed with NaCl aqueous solutions of CaCl$_2$ and WP. Dispersions were equilibrated overnight at room temperature and used after pH adjustment with 7.5% (w/w) lactic acid solution. Sodium azide [0.02% (w/w)] was added to avoid bacterial growth. Dry matter was controlled by drying each sample overnight 103 °C.

NMR Measurements. Samples of 0.5 mL were taken (0.5 mL equivalent to 10 mm height corresponding to the homogeneous area of the probe, 8 mm diameter) and put into sealed NMR tubes. Measurements were carried out with a 0.47 T NMR spectrometer (PC 120, Bruker, Wissenbourg, France) operating at 20 MHz for protons at 40 °C. Two kinds of NMR sequence were used: (i) free induction decay (FID) and (ii) Carr−Purcell−Meiboom−Gill (CPMG). The short relaxation times (11 μs < T_2 < 100 μs) were detected from the rapid part of the FID signal sampled between 11 and 70 μs with a 1 MHz acquisition card (Team 490, Bakker Electronics, Ba Dongen, Netherlands). The recuperation delay (RD) was 10 s for 3% NPC dispersions, 5 s for 9% NPC dispersions, and 4 s for 15% NPC dispersions). Long relaxation times (T_2 > 1 ms) could not be measured with this sequence because of field heterogeneity. They were measured with a CPMG sequence composed of 845 points. The pulse spacings, (τ) between the 90° and 180° pulses were 1300, 350, and 150 μs, and RD were 10, 5, and 4 s for 3, 9, and 15% NPC dispersions, respectively. NMR measurements were repeated three times for each sample.

NMR Decay Curve Analysis. The NMR decay curve fitting methods proposed in the literature can be divided into two groups: discrete methods and continuous methods. The most commonly used discrete method used is the Marquardt method (*36*). The main disadvantage of discrete methods is the prior need to know the number of relaxation components, and accuracy depends on the entry parameters. In contrast, continuous methods such as the maximum entropy method (MEM) (*37*) do not require any assumption of the number of components.

We therefore decided to analyze the CPMG sequence with the maximum entropy method first in order to determine the number of relaxation components and then, according to the results, a model was defined to fit the data with the Marquardt method. This procedure was chosen because the accuracy of the Marquardt method is better than that of the MEM when the number of components is small. The model validated by the MEM was a biexponential function:

$$I(t) = I_{cp1}e^{(-t/T_{2cp1})} + I_{cp2}e^{(-t/T_{2cp2})} + cte \quad (3)$$

with $I(t)$ the intensity of the NMR signal at given t and $I_{CPi1,2}$ and $T_{2CP1,2}$ the intensity and the spin−spin relaxation time of each relaxation component, respectively.

For fitting using the Marquardt method, we took into account the NMR signal measured after application of the pulse to an empty tube. Even so, the intensity of the signal represented 126 mV, corresponding to electronic noise due to ringing in the transmitter. The signal intensity measured was compared to the expected signal intensity deduced from

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Table 2. T_2 Relaxation Parameters Obtained for Milk and 3, 9, and 15% NPC Dispersions

	NPC 3[a]	NPC 9[a]	NPC 15[a]	milk
T_{2CP1} (µs)	0.80–9.86	19 ± 0.4	9.6–133	
I_{2CP1} (%)	1.5–3.7	6.7 ± 0.3	9.1–10.4	
T_{2CP2} (ms)	162.8–203.5	53.2 ± 0.3	25.0–30.1	164.2 ± 0.5
I_{2CP2} (%)	96.3–98.5	93.3 ± 0.3	89.6–90.9	100

[a] Values correspond to minimum and maximum values for 3 and 15% NPC dispersions and to mean ± standard error for 9% NPC dispersions.

the composition of the sample. The expected signal intensities can be calculated from the chemical composition corrected by the mass intensity (MI) expressed in volts per gram from each protonated compound. The expected mass intensity was calculated from the MI of each milk component containing protons (lactose, water, and proteins) according to

$$\frac{MI^{pur}_{comp} m_{comp}}{m_{samp}} = MI^{exp}_{comp} \qquad (4)$$

with MI^{pur}_{comp} the mass intensity of the pure component at measurement temperature (40 °C), m_{comp} the content of one component in the sample (g), and m_{samp} the sample weight.

Statistical Analysis. The effect of sample composition on relaxation time and amplitude was quantified using a 2^{5-1} experimental factorial design with five factors (milk components and pH) and three levels (**Table 1**). This incomplete factorial design was constructed to analyze the effect of each factor independently and also the first-order interactions. The second-order interactions were not considered in this way, and the number of experiments was reduced. The central point (level 0) was repeated five times.

RESULTS AND DISCUSSION

Characterization of NMR Signals. The NMR parameters determined with the Marquardt method for milk and for 3, 9, and 15% NPC dispersions are given in **Table 2**.

The fitting model was the same for each sample composition, that is, two exponential components for the rapid and slow relaxation components (CP1 and CP2), respectively.

The relative intensity of the first component (I_{CP1}) was still very weak, especially for the 3% NPC concentration. For a higher NPC concentration (15%), this intensity increased to 10% of the total signal, and its relaxation time (T_{2CP1}) varied from 0.8 to 10 ms according to soluble phase composition. These results could be explained by the instability of the signal fitting because the intensity was too low. This component has rarely been described in the literature.

The second component (CP2) was characterized by a higher relative intensity (I_{CP2}), that is, >90% of total signal. The corresponding relaxation times (T_{2CP2}) and intensity decreased with the NPC fraction. For 3% NPC concentration, the relaxation time was similar to that of milk (≈164 ms for milk and 162.8–203.5 ms for NPC dispersions). Values obtained for milk were consistent with published findings for similar experimental conditions (skimmed milk powder at 9.3% and pH 6.6) (*9, 16*). However, only one relaxation component was found for milk relaxation compared to the NPC solution, for which two relaxation components were obtained. This component in milk was attributed to water protons and to exchangeable protons of solutes (*9*). Nevertheless, this has not previously been quantitatively validated in a complex mixture. These results suggest that the first component is related to NPC protons and the second to water protons. To validate this hypothesis, experimentally measured signal intensities expressed in volts per gram of sample
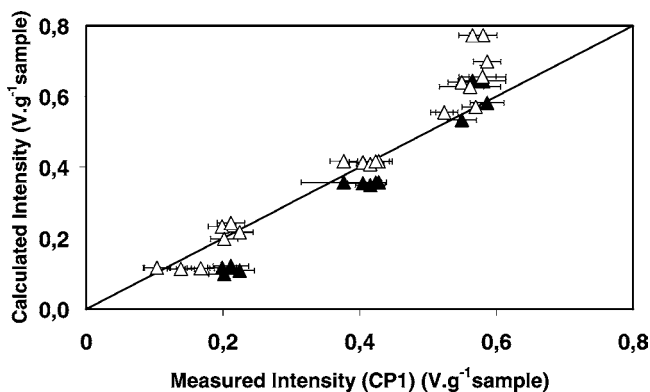

Figure 1. Comparison between experimental NMR signal intensity (V·g⁻¹) for CP1 and expected signal intensity according to NPC content (▲) and NPC + whey protein content (△). The $Y = X$ slope corresponded to an ideal situation for which experimental and expected intensities were similar.
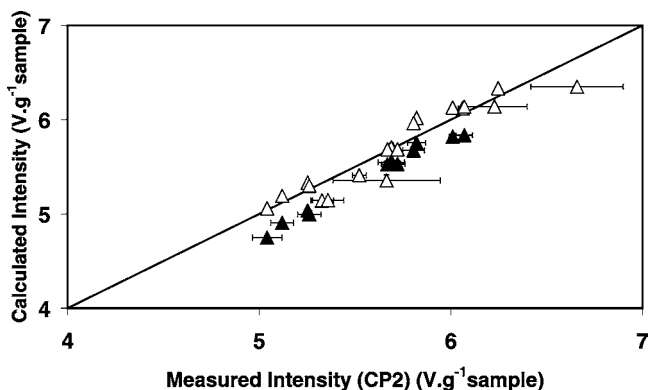

Figure 2. Comparison between experimental NMR signal intensity (V·g⁻¹) for CP2 and expected signal intensity according to water content (▲) and water + lactose content (△). The $Y = X$ slope corresponded to an ideal situation for which experimental and expected intensities were similar.

were compared to calculated intensities deduced from the chemical composition of dispersions. The relationships between measured and calculated intensities obtained for each relaxation component (CP1 and CP2) are given in **Figures 1** and **2**. Independently of pH, I_{CP2} measured by NMR was similar to the sum of calculated intensities of water and lactose. This confirmed that this component could be attributed to water and lactose protons. On the other hand, the attribution of CP1 to NPC and WP depended on NPC concentration. For 3−9% NPC concentrations, the amount of nonexchangeable NPC proton was sufficient to explain the intensity of the first component (CP1). For 15% NPC concentration, the measured CP1 intensity was in agreement with the intensity expected if only nonexchangeable protons from NPC were considered. For lower concentrations, the protons from WP should be taken into account. This effect could be explained by the signal fitting. When the signal intensity was low, the fitting method tended to reduce the expected signal intensity artificially, with an overestimation of the relaxation time value. The spin−spin relaxation times estimated for nonexchangeable protons are generally lower than those obtained in this experiment (*38*). Consequently, misadjustment of this component can be suspected, and the variations in relaxation time parameters from CP1 are not discussed below.

Dispersion Composition Effect on NMR Signal Relaxation Rate of Water. The results of the variance analysis of the experimental design for relaxation rate R_{22} are given in **Table 3**. A predominant influence of NPC concentration, which tended

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Table 3. Variance Analysis for Water Proton Relaxation Rate (= $1/T_{2CP2}$) (99% Confidence Interval)[a]

factor	F ratio	P value
A: NPC	23086.1	****
B: WP	46.65	****
C: CaCl$_2$	6.63	ns
D: pH	64.83	****
E: lactose	87.71	****
AB	34.33	****
AC	1.31	ns
AD	39.71	****
AE	27.96	****
BC	14.74	ns
BD	0.39	ns
BE	0.1	ns
CD	0.18	ns
CE	1.39	ns
DE	11.77	ns
lack of fit	394.11	0

[a] Fischer coefficients (F ratio) are given. ns, nonsignificant factors. The model explained 99.74% of variations observed.
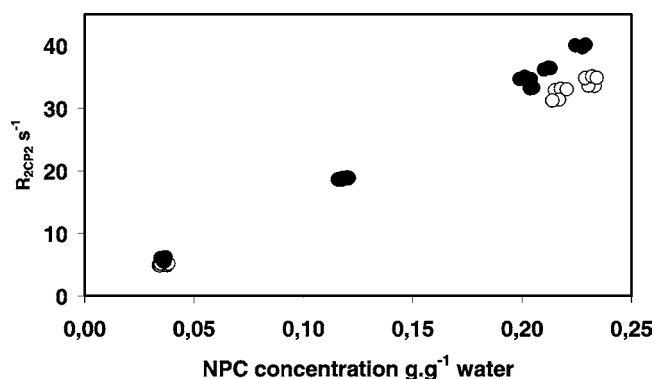
Figure 3. Variations in R_{2CP2} according to NPC concentrations (g/g of water) without lactose (○) and with lactose (●).

Figure 4. Variations in R_{2CP2} according to NPC concentrations (g/g of water) and pH values: pH 6.6 (shaded circles); pH 6.4 (white circles); pH 6.2 (black circles).

Figure 5. Variations in R_{2CP2} according to NPC concentrations (g/g of water) without WP (○) and with WP (●).

to increase the water relaxation rate, was observed. The influences of pH, lactose, and WP were also significant, but to a lesser extent than NPC. The influence of casein micelles on the relaxation rate of water has already been observed during rehydration of NPC powder (*39*). Our results clearly demonstrated that NPC concentration can explain most of the relaxation rate variations, despite changes in the concentrations of lactose and whey protein). However, addition of lactose tended to increase the relaxation rate of the aqueous phase (**Figure 3**). This effect was more significant for a high NPC concentration (15% NPC) and explained the significant interaction between lactose and NPC (**Table 3**).

Lowering the pH involved a decrease in the relaxation rate of water (**Figure 4**). As demonstrated by the significant interaction detected by the variance analysis, this effect was dependent on the NPC concentration (**Table 3**). Moreover, the influence of the pH on water relaxation rate was more pronounced without lactose. The decrease in relaxation rate achieved by lowering pH was less in the presence of lactose. Whey proteins also tended to increase the water relaxation rate (**Figure 5**), especially for dispersions without lactose and high NPC concentrations, but this effect was less pronounced than for lactose and pH. The addition of CaCl$_2$ had no effect on the water relaxation rate. This was in agreement with previous results demonstrating that addition of CaCl$_2$ did not lead to major changes in the micellar structure or in casein hydration (*11*). Almost all of the added calcium remained in the soluble phase.

Interpretation of the Water Relaxation Rate. Although NMR relaxation models (see Theory) are appropriate to explain the observed water relaxation of milk, they have never been evaluated on mixed milk protein dispersions. Exploitation of these findings should provide validation, in two steps: (i) by considering the effects of NPC and pH on water relaxation rate and (ii) by taking into account other solutes such as WP and lactose.

Dispersions without Lactose and WP. Equation 2 should be valid to explain the variations in relaxation rate in a pure NPC dispersion. Indeed, because the chemical exchange mechanisms are mainly attributable to the exchangeable protons from lactose and soluble protein, they can be ignored in pure NPC dispersion. Moreover, the heterogeneity of micellar structure is too small (between 180 and 200 nm) (*40*) to cause multiexponential behavior. The slow diffusive exchange condition between extramicellar and intramicellar water molecules is respected only if the limit $a^2\Delta\gamma/D > 1$ is correct (*8*). $\Delta\gamma$ is the difference between the relaxation rates in the two water compartments, and D is the water self-diffusion coefficient. Assuming $a = 150$ nm, $T_{2f} = 2$ s, and $D = 2.5 \times 10^{-9}$ m$^2 \cdot$s^{-1}, the relaxation time of water protons in interaction with casein should be <3 μs. Such small values are improbable for water molecules. This means that water relaxation inside the casein micelle should be in fast diffusive exchange with the bulk water. We therefore analyzed the water relaxation rate according to NPC concentration with a simple linear regression (eq 5).

$$R_{2obs} = R_{2a} + nC_{casein}(R_{2b} - R_{2a}) \qquad (5)$$

The slope is $n(R_{2b} - R_{2a})$, and the intercept of the x axis is equal to R_{2a}. **Figure 6** demonstrates that the linear regression was validated in the NPC concentration range of the study. Moreover, the findings were in complete agreement with the
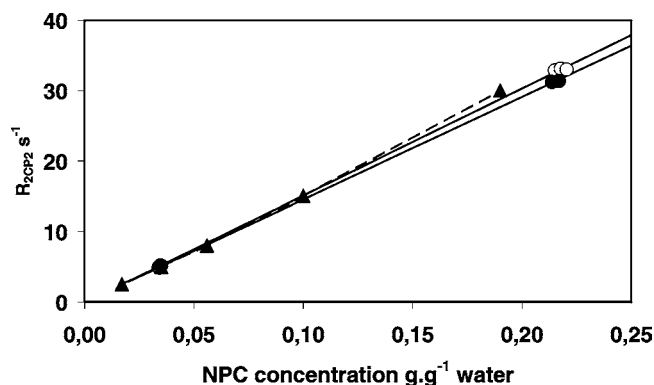
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Figure 6. Variations in R_{2CP2} according to NPC concentrations (g/g of water) at pH 6.6 (○) and pH 6.2 (●). Data from Davenel et al. (*38*) are included (▲).

Table 4. Regression Parameters Calculated for Relation $R_{2CP2} = aX_{NPC} + b$[a]

dispersion	a (slope)	b (y intercept)	r^2
LAC-, WP-, pH 6.2	145.7 ± 0.6	−0.05 ± 0.09	0.999
LAC-, WP-, pH 6.6	152.2 ± 1.1	−0.14 ± 0.18	0.999

[a] Only dispersions with no lactose and no whey proteins were considered.

results of Davenel et al. (*39*). Parameters calculated for dispersions at pH 6.2 and 6.6 are given in **Table 4**.

The values of the intercept of the y axis were <0.3 s⁻¹ (free water proton relaxation rate at 40 °C). Nevertheless, the standard errors calculated for this parameter were wide and might be reduced by increasing the number of experiments. The slope value varied with pH, that is, from 145.7 ± 0.6 s⁻¹g·g⁻¹ at pH 6.2 to 152.2 ± 1.1 s⁻¹g·g⁻¹ at pH 6.6. These results could be attributed to two mechanisms: (1) a variation in hydration number *n* and (2) a modification of the hydration water relaxation rate R_{2b}.

The influences of pH on hydration and voluminosity of casein micelles have been widely studied. Decreasing pH tends to reduce hydration of casein micelles (*10, 41−44*). These studies were carried out at different temperatures (20−37 °C) and showed similar hydration profiles. This suggests that variations in hydration with pH were slightly influenced by temperature. Hydration rates calculated at pH 6.2 and 6.6 were 2.7 and 2.3 ± 0.1 g of water per gram of casein micelle, respectively (*10*). Hydration measurements were also performed for NPC and showed a difference of 4% between pH 6.2 and 6.6 (*28*).

Variations in hydration were consistent with the slope variations observed in this study (a loss of 4.7% between pH 6.2 and 6.6). We can therefore assume that the decrease in the slope versus pH was explained by only a change in hydration, and thus the water relaxation rate should be considered as independent of pH despite a structural change. In view of these results, and assuming a hydration value of 2.4 g·g⁻¹, the hydration water relaxation rate was estimated at 63.4 ± 0.5 s⁻¹ according to eq 5. These results could be explained by the fact that for small molecules the hydration water visualized by NMR was attributed to water molecules, the mobility of which is affected by interactions between polar groups of these molecules and other molecules (hydrogen and electrostatic interactions). This water molecule fraction is very low and does not exceed the first monolayer at the protein surface. The water molecules inside the micelle should therefore be divided into two fractions: a large fraction attributable to bulk water inside and a smaller fraction attributable to the first monolayer.

Table 5. Influence of Layer ϵ on Intramicellar Water Relaxation Times T_{2s} for Different pH Values[a]

ϵ (Å)	T_{2s} (ms) − pH 6.6 (2r = 210 nm)	T_{2s} (ms) − pH 6.2 (2r = 200 nm)
25	1.12	1.06
20	0.9	0.94
10	0.45	0.47

[a] Variations in the micelle radius *r* were considered according to pH.

According to the relaxation model proposed to describe the relaxation in porous media, the water relaxation $1/T_{2b} = R_{2b}$ inside the micelle deduced from the hydration or voluminosity of the micelle is given by

$$\frac{1}{T_{2b}} = \frac{1}{T_{2f}} + \frac{S\epsilon}{VT_{2s}} \qquad (6)$$

with $1/T_{2f}$ the free water relaxation rate and $1/T_{2s}$ the relaxation rate of water in close interaction with the casein micelle on layer ϵ (*45*).

Measurement of the diffusion coefficient and simulation of the molecular dynamics of water on the protein surface (proteins such as lysozyme) may suggest a layer of 10−15 Å of water with a diffusion coefficient lower than that of "free" water (*46*). We assumed that a water molecule in close interaction with proteins shows similar behavior, independently of its structure and composition, and thus the same value of ϵ was used for casein micelles. We considered casein micelles as a spherical [surface (S)/volume (V) = 6/2r (radius)], and the relaxation time of water in close interaction with casein became

$$T_{2s} = \frac{\epsilon}{\dfrac{1}{T_{2s}} - \dfrac{1}{T_{2f}}} \times \frac{6}{2r} \qquad (7)$$

The relaxation times calculated for different layers ϵ are presented in **Table 5**. The average diameter was chosen according to published data obtained on NPC dispersions (200 and 210 nm for pH 6.2 and 6.6, respectively) (*28*). The relaxation time for water molecules in the first monolayer obtained at 1 ms was consistent with the values usually attributed to water in close interaction with casein (*8, 19*).

Dispersions with Lactose and WP. As shown in **Table 3** and **Figure 3,5**, the influence of lactose and whey protein on water relaxation rate was clearly demonstrated. The presence of lactose and whey protein in dispersions increased the water relaxation rate. This phenomenon was particularly pronounced in highly concentrated NPC dispersions. This effect could be explained by the following.

(1) A decrease in the correlation time of NPC was induced by an increase in the viscosity of the bulk solution in the presence of lactose. If the correlation time decreased, then the exchangeable proton relaxation would decrease and this effect would therefore be transferred to water protons by chemical exchange.

(2) There was a direct effect of lactose or whey protein on water relaxation. For example, a large amount of water was inside the micelle at high NPC concentrations, and the remaining water content available for lactose hydration decreased. The lactose/water ratio was 7.5 g/100 g, whereas it should be equal to 11.7 g/100 g, assuming casein hydration at 2.4 g·g⁻¹.

(3) An increase in casein hydration occurred. Effects of adding sugars such as sucrose or lactose to casein solution on casein hydration have already been reported (*26, 27*). ¹⁷O NMR and

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^{2}H NMR relaxation studies demonstrated that the "trapped" water hydration (in g/g) increased from 0.00452 to 0.00830 when 300 mM lactose was added to a bovine caseinate solution. Although the NPC concentration range was different between our study and that of Mora-Guttierrez et al. (*26*), the same effect may have occurred. Recently, Schorsch et al. (*47*) investigated the effect of sucrose addition on the formation of casein gels. They found that at high sucrose levels the micelle structure is more swollen due to a greater hydration.

(4) There was a change in the micelle structure. An unfolding influence of lactose toward casein has been reported during lactose crystallization in frozen products (*48, 49*). This hypothesis could not be considered for two reasons: first, because a hysteresis phenomenon has been observed for lactose. A crystal of rehydrated lactose cannot be crystallized again if it is dehydrated again. Moreover, the concentrations of lactose in 15% dispersions were not high enough (20 g of lactose/g of water) at 40 °C to be crystallized.

Conclusion. In this experiment two relaxation components from NMR signals were observed and attributed to water and NPC protons in various dispersions simulating milk composition, respectively. However, the accuracy on the first component (CP1) must be increased to reveal structural or composition effects.

The second component was attributed to proton relaxation from the aqueous phase in the casein dispersion. This finding would be particularly interesting to follow water behavior at each stage of transformation of milk (coagulation, syneresis, and draining). Despite relatively high dilutions (>70% water content) and the complexity of the media studied, the behavior of water was mainly affected by NPC concentration. These results are compatible with conclusions observed in different studies carried out with poorly hydrated binary milk protein mixture (<20%) obtained with powders (*7, 15, 50*).

The effect of casein concentration on the water relaxation rate was described with the Fedotov relaxation model (*33*). This model integrates the participation of exchangeable protons of macromolecules in water relaxation. In the case of NPC dispersions without lactose, this model may be simplified and provides a good explanation for the water relaxation related to the casein micelle concentration. Moreover, pH action can be integrated in this model. The variations in hydration observed between pH 6.6 and 6.2 (*28*) were consistent with slope variations observed by NMR.

Two fractions of hydration water could be distinguished from the model describing water relaxation in porous media. The first fraction corresponded to water in fast diffusive exchange. This water diffused inside the micelle and exchanged with extra-micellar water. The amount measured might therefore be directly linked to micelle structure. The second fraction corresponded to water in close interaction with protein. The amounts measured for this second fraction were lower and were consistent with those usually measured for the monolayer (sorption isotherm) (*1*). In contrast to "free" water, this water would be not sensitive to variations in pH and, therefore, to micelle structure.

We demonstrated here that interactions should be considered to explain the water relaxation rate in the complex casein dispersion. For example, addition of lactose to a 15% NPC dispersion induced an increase in the water relaxation rate that was not observed for more diluted dispersions. This effect was interpreted as an increase in the viscosity of the water phase and an increase in casein hydration.

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