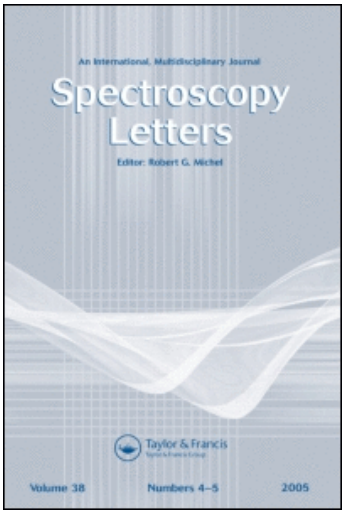

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NMR Solvent Spin-Lattice Relaxation Rate in Colostrum

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Abstract: In this study, by using a FT-NMR spectrometer operating at 60 MHz for proton, the solvent spin-lattice relaxation times (T_1) in colostrum were measured versus the days of lactation, whereas the T_1 values in dehydrated colostrum were determined versus concentration of its hydrating solid. Data show that the spin-lattice relaxation rate ($1/T_1$ or R_1) in colostrum is linearly dependent upon the inverse of time ($1/$days), and the R_1 in dehydrated colostrum increases linearly with increasing concentration of its hydrating solid content (C). From data, the total paramagnetic contribution of ions in colostrum to the R_1 was found to be negligible. The dehydrated colostrum data indicates that the R_1 in colostrum is linearly dependent upon its hydrating solid content. Therefore, the R_1 changes in colostrum were analyzed in terms of the relaxivities (increase in relaxation rate per unit concentration of solid) and the concentrations of milk constituents. Such an analysis provides a relation similar to that of the R_1 in dehydrated colostrum. The current data imply that the relaxation changes in colostrum by days may be explained through changes in the concentrations of milk constituents. Also, the data suggest that the relaxation mechanism in colostrum can be explained in terms of fast chemical exchange of protons between free water and water bound to milk constituents.

Keywords: Carbohydrate, colostrum, milk, NMR, protein

INTRODUCTION

During the first few days after birth, a woman's body produces a fluid called colostrum. Colostrum has more protein and minerals, but less fat and sugar,

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compared to the mature milk. Colostrum secretion lasts about 6 days and changes to mature milk gradually.[1–3] The breast milk is the best source of nutrition for an infant. Breast milk can provide all or nearly all the nutrition an infant needs during the first 6 to 12 months of life.[4,5] Therefore, several studies have been carried out on the changes in the composition of women's milk for period of 6 months after birth. These studies have revealed that the composition of milk is dependent on the days of lactation period. On the other hand, high-resolution NMR and other techniques have recently been used to record NMR spectrum of colostrum and mature milk and to determine the changes in some milk constituents during lactation period.[6–9] However, the peak intensities were used for the evaluation of the changes. To the best of authors' knowledge, the changes in spin-lattice relaxation rate of milk during lactation period have not been studied yet.

In this study, the spin-lattice relaxation time (T_1) in colostrum was measured versus days after birth. To evaluate factors responsible for changes in the spin-lattice relaxation rate ($1/T_1$ or R_1), the R_1 in dehydrated colostrum was determined versus its hydrating material content. In addition, the experiment was extended to mature milk in order to determine the dependence of the R_1 in milk on the inverse of time ($1/\text{days}$). The relaxation mechanism in colostrum was also explained.

EXPERIMENTAL

Preparation of Samples

Seven samples of colostrum (1–5 days) and 10 samples of mature milk (from day 20 to 6 months) were collected from healthy breast-feeding mothers. Of these samples, 1.5 mL was used for NMR T_1 measurements versus days after birth.

A sample of the third day's colostrum was divided into three parts. One part was used for dehydration process and relaxation measurements, while the others were used for the determinations of total fat and total solid in the sample. Initial concentration of the fat was determined by creamatocrit method,[10,11] whereas total solid content of the colostrum was determined by weighing the sample before and after drying it at 120°C for 10 hr. The colostrum specific gravity (mass/volume) was found to be $1.04\,\text{g/cm}^3$ and used to convert the weight of milk to its volume. In dehydration experiment, the difference between total solid and fat was described as initial content of total hydrating solid. The T_1 in the sample corresponding to the initial concentration was measured as a first step. In the second step, the sample was exposed to an air flow for 3 hr, weighed, and then the amount of the dehydrated milk was determined. Finally concentration of the hydrating solid content and the corresponding relaxation time were determined. The dehydration procedure was repeated as in the second step, as long as NMR signal was reliable for

relaxation measurements. The R_1 values were plotted versus corresponding concentrations.

T_1 Measurements

Relaxation measurements were carried out on a JEOL FX (Tokyo, Japan) 60Q FT-NMR spectrometer operating at 60 MHz for proton, and 10 mm OD NMR tubes were used. The inversion recovery pulse sequence (180°-τ-90°) was used with delay time τ varying from 0.2 to 5 s. The pulse repetition time was set at 20 s. The magnetization decay curve was found to be a single exponential. Probe temperature was kept at (20°C $\pm$ 0.5°C), by using a VT-3C automatic temperature controller unit (JEOL LTD, Tokyo, Japan).

Paramagnetic Contribution of Ions to the Relaxation Rate of Colostrum

Mothers' milk contains small amounts of iron and copper, which are paramagnetic.[12] Ascorbic acid is a reductant for these ions, and addition of ascorbic acid to a milk sample reduces the iron and copper to diamagnetic form.[13] The paramagnetic contribution is defined as a difference between the observed relaxation rates of a solution in the presence and absence of ions.[13,14] The paramagnetic contributions of reduced iron and copper are negligible and therefore, the solution containing such ions can be considered without paramagnetic ions. The R_1 in a colostrum sample was measured before and after addition of ascorbic acid. The difference in R_1 was used as total paramagnetic contribution of the ions to the colostrum. However, if the ions are bound, ascorbic acid may not be effective for reduction. When pH of solution is reduced to lower values, the ions are fully dissociated and their paramagnetic contributions increase.[13,14] Taking into consideration this point, the pH of a sample was reduced to 2 by using a small amount of phosphoric acid. The R_1 of this sample was measured before and after addition of ascorbic acid, and the total paramagnetic contribution at pH = 2 was determined.

Proton Relaxivity

The R_1 values in both colostrum and mature milk versus inverse of time were analyzed in terms of individual proton relaxivities and concentrations of hydrating materials in milk. Therefore, it should be useful to give the definition of the proton relaxivity. The proton relaxivity of a protein solution can be defined as the incremental increase in relaxation rate per unit concentration of protein.[15,16] In the presence of different solids in a solution, the

relaxivity of solution can be averaged by using individual relaxivities and fractional contents of the solids.[16,17]

RESULTS AND DISCUSSION

The changes in the relaxation rate of breast milk were represented by R_1 versus inverse time. Figure 1 shows the dependence of the relaxation rate in colostrum on the inverse time after birth, whereas Fig. 2 shows the least squares fit of the relaxation rate in dehydrated colostrum versus concentration of its hydrating solid content. As seen from Figs. 1 and 2, the R_1 in colostrum is linearly proportional to the inverse time, and the R_1 in the dehydrated colostrum is linearly dependent upon the concentration of its hydrating solid content (C). Furthermore, the paramagnetic contributions of the ions at physiological pH and pH = 2 are shown in Table 1. It is seen that the total paramagnetic contribution of the ions to the R_1 in colostrum is negligible.

In the dehydration experiment, the concentration of total solid material in milk was increased only by evaporating water. It means that the dehydrated colostrum is similar to colostrum except gradually increasing material content. Therefore, the dehydrated colostrum data indicates that the R_1 in colostrum is dependent upon its hydrating solid content. This is consistent with the R_1 data in other biological fluids, as the R_1 in such fluids was found to be proportional to the material content.[15–21] For these reasons, the changes in the R_1 caused by inverse time should be related to the changes in colostrum composition during lactation period. This point can be confirmed by the following analysis.

Colostrum is the deep yellow-colored milk secreted 6 days after birth. Colostrum can be considered a mixture of water and solid materials. Solid materials consist of carbohydrates, proteins, vitamins, milk lipids, amino acids, and minerals. The milk constituents other than fat can bind water, and therefore, they were described as hydrating solid material in this work.

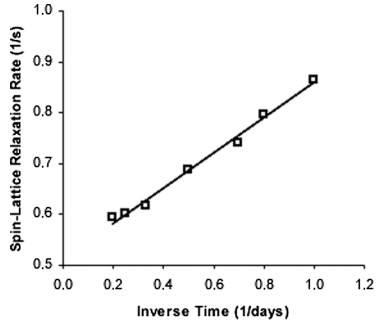

Figure 1. Dependence of the spin-lattice relaxation rate in native colostrum on the inverse time of lactation.

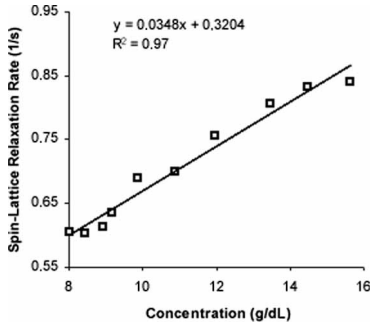

Figure 2. The spin-lattice relaxation rate in dehydrated colostrum versus hydrating solid content.

This expresses that each of carbohydrates, proteins, vitamins, amino acids and minerals in colostrum has its own relaxivity. Thus, the relaxation rate in colostrum can be considered:[16,17]

$$\frac{1}{T_1} = \frac{1}{T_{1w}} + C_{\text{chyd}}\, R_{\text{chyd}} + C_{\text{prot}}\, R_{\text{prot}} + C_{\text{vit}}\, R_{\text{vit}} + C_{\text{aa}}\, R_{\text{aa}} + C_{\text{min}}\, R_{\text{min}} \qquad (1)$$

where subscripts "w", "chyd", "prot", "vit", "aa" and "min" represent free water, carbohydrate, protein, vitamin, amino acids, and mineral, respectively, and C denotes the related concentrations. However, the concentrations of vitamins and amino acids in milk are very low[1] and the contributions of minerals and other ions are negligible. For these reasons, the main hydrating solids contributing to the R_1 in colostrum can be considered carbohydrates and proteins. Hence, Eq. (1) aproximates to

$$\frac{1}{T_1} \approx \frac{1}{T_{1w}} + C_{\text{chyd}}\ R_{\text{chyd}} + C_{\text{prot}}\ R_{\text{prot}} \qquad (2)$$

Table 1. Total paramagnetic contribution of iron and copper in colostrum at physiological pH and pH = 2, in units of s^{-1}

Colostrum					
pH = 2			pH ≈ 7		
$1/T_{1b}$	$1/T_{1a}$	$\Delta 1/T_1$	$1/T_{1b}$	$1/T_{1a}$	$\Delta 1/T_1$
0.644	0.630	0.014	0.610	0.603	0.007

The difference between the relaxation rates measured before and after addition of ascorbic acid ($\Delta 1/T_1$) was considerd as the total paramagnetic contribution.

Equation (2) should explain the relaxation changes by the inverse time since concentrations of colostrum constituents change during secretion.[1] In fact, the mean concentrations of carbohydrate and protein in colostrum were given to be 5.7 and 2.3 (g/dL), respectively.[1] For the mean values, Eq. (2) can be written as

$$\frac{1}{T_1} \approx \frac{1}{T_{1w}} + (5.7\,\mathrm{g/dL})R_{chyd} + (2.3\,\mathrm{g/dL})R_{prot} \tag{3}$$

On the other hand, the relation in Fig. 2 representing dehydrated milk can be expressed as follows

$$\frac{1}{T_1} = 0.32 + 0.0348C \tag{4}$$

where R_1, 0.32, and 0.0348 are the observed relaxation rate, the relaxation rate of free water and the slope of relation, respectively. The slope in Eq. (4) represents the relaxivity of total hydrating solid in dehydrated milk, which is similar to that of serum total protein (TP), being about 0.04 $(\mathrm{g/dL})^{-1}\,\mathrm{s}^{-1}$.[16] This similarity implies that the total protein relaxivity in milk (R_{prot}) should be nearly equal to that of serum TP. Then, from Eq. (3), the contribution of total protein in collostrum to the R_1 and R_{chyd} can be calculated as $0.092\,\mathrm{s}^{-1}$ and 0.03 $(\mathrm{g/dL})^{-1}\,\mathrm{s}^{-1}$, respectively. Because the $R_{prot}/R_{chyd} = 1.3$ and $R_{av}/R_{chyd} = 1.15$, both relaxivities in Eq. (3) can be replaced by the average (R_{av}) of R_{chyd} and R_{prot}, which is about 0.035 $(\mathrm{g/dL})^{-1}\,\mathrm{s}^{-1}$. Then, Eq. (3) reduces to

$$\frac{1}{T_1} \approx \frac{1}{T_{1w}} + 0.035(5.7\,\mathrm{g/dL} + 2.3\,\mathrm{g/dL}) = \frac{1}{T_{1w}} + 0.035C \tag{5}$$

where C is total mean concentration of hydrated solid contributing to the R_1. Equation (5) derived from Eq. (2) is very similar to the relation in Fig. 2 concerning dehydrated colostrum.

The experimental relation in Eq. (4) represents the dependence of the R_1 on the concentration of total hydrating solids in colostrum. The similar relation was derived from Eq. (2) by using the mean concentrations of protein and carbohydrate in colostrum. This similarity emphasizes that the changes in the R_1 by inverse time are essentially due to the changes in colostrum composition. This was further confirmed by inserting the concentrations of carbohydrates (6 g/dL) and total protein (3 g/dL) in two-days colostrum into Eq. (2). In this case, the R_1 of the sample was reproduced as $0.65\,\mathrm{s}^{-1}$, which was measured as $0.68\,\mathrm{s}^{-1}$ experimentally. This analysis is also consistent with the previous NMR study on peak assessment of time variation in composition of human milk and colostrum, as peak intensity is related to the concentration of corresponding milk constituent.[6]

Colostrum and mature milk can be considered as two different stages of lactation period. Colostrum contains high protein and less carbohydrate than

mature milk and it transforms to mature milk gradually. Mature milk composition is known to change from the beginning of a breast feeding session to the end of a session. Taking into consideration this point, the relaxation measurements were extended to the lactation period corresponding to mature milk. The data is shown in Fig. 3. It is seen that R_1 in mature milk is also linearly dependent on inverse time. The similarity between graphs of colostrum (Fig. 1) and mature milk (Fig. 3) implies that the analysis based on colostrum composition should apply to mature milk.

Equation (4) and Eq. (5) indicate the fast chemical exchange of protons between free and bound water. In fact, the relaxation rates in biological fluids are known to be averaged by population-weighted rapid chemical exchange of water protons between free and bound states. This can be expressed as follows:[22]

$$\frac{1}{T_1} = \frac{P_f}{T_{1f}} + \frac{P_b}{T_{1b}} \tag{6}$$

where P_f, P_b, R_{1f}, and R_{1b} refer to the fractions and the relaxation rates of free and bound water, respectively. Inserting $P_f = (1 - P_b)$ into Eq. (6) gives

$$\frac{1}{T_1} = \frac{1}{T_{1f}} + P_b\left(\frac{1}{T_{1b}} - \frac{1}{T_{1f}}\right) \tag{7}$$

Because $R_{1f} \ll R_{1b}$,[22] Eq. (7) reduced to Eq. (8):

$$\frac{1}{T_1} = \frac{1}{T_{1f}} + P_b\left(\frac{1}{T_{1b}}\right) \tag{8}$$

Equation (8) can be derived in a different way; Because $P_b \ll P_f$ in biological fluids,[22] P_f can be nearly taken as 1, and then Eq. (6) becomes identical to Eq. (8). On the other hand, the fraction of bound water is proportional to the solid content.[22,23] Therefore, P_b can be written as $P_b = LC$, where L is

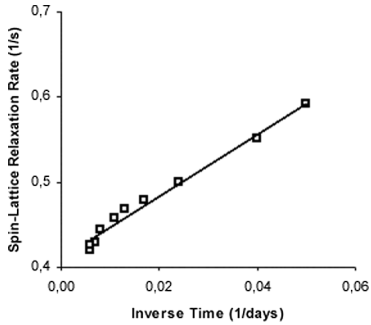

Figure 3. Dependence of the spin-lattice relaxation rate in mature milk on the inverse time of lactation.

proportionality constant. Then, Eq. (8) can be written as

$$\frac{1}{T_1} = \frac{1}{T_{1f}} + \left(\frac{L}{T_{1b}}\right)C \tag{9}$$

if we define L/T_{1b} as K, Eq. (9) becomes

$$\frac{1}{T_1} = \frac{1}{T_{1f}} + KC \tag{10}$$

where R_{1f} and K represent the relaxation rate of free water and the slope of the relation, respectively. Equation (10) is identical to the relations in Eqs. (4) and (5). This identification shows that the R_1 in colostrum is caused by fast chemical exchange of water protons between free and bound water. This identification is also consistent with Eq. (1), implying the fast chemical exchange between different environments.

Equation (8) may be used for determinations of bound water fraction and the amount of bound water per gram of water binding molecules. This requires the calculation of the relaxation rate of bound water (R_{1b}) in colostrum. Such a calculation can be done if one assumes an average rotational correlation time for all water binding molecules in colostrum. The relaxation rate of bound water in a biological sample is known to be caused by dipole–dipole interaction of protons, and it can be expressed by Solomon–Blombergen equation for paired interaction.[24] The Solomon–Blombergen equation for bound water can be written as

$$\frac{1}{T_{1b}} = \frac{3}{10}\frac{\hbar^2\gamma^4}{r^6}\left[\frac{\tau}{(1+\omega^2\tau^2)} + \frac{4\tau}{(1+4\omega^2\tau^2)}\right] \tag{11}$$

where $\hbar$, γ, r, τ, and ω are the gyromagnetic ratio for proton, Planck's constant divided by 2π, dipole–dipole distance, correlation time, and resonance frequency, respectively. Multiplication of factors in front of the parenthesis in Eq. (11) can be calculated as 1.02×10^{10} by taking r value as 1.58 Å. An average value of $13.5\,s^{-1}$ for the R_{1b} in colostrum can be calculated by using 10^{-8} second for the average τ value of rotationally bound water[23–26] and 60 MHz for the resonance frequency. Then P_b can be calculated as 0.028 by inserting the average relaxation rates of the colostrum samples ($0.7\,s^{-1}$) and free water ($0.33\,s^{-1}$), and the R_{1b} into Eq. (8). Thus, the amount of bound water may be calculated as 0.32 g per gram of water binding solid. This is consistent with previous results obtained for albumin solutions, being about 0.4 g per gram of dry albumin.[27] However, this calculation gives a rough idea only for bound water fraction in colostrum. An exact calculation requires a separate τ value for each type of water binding molecules in milk.

In conclusion, the current data imply that the relaxation changes in milk (both colostrum and mature) may be explained by changes in concentrations of milk constituents. Also, the data suggest that the relaxation mechanism in

milk can be explained in terms of fast chemical exchange of protons between free water and water bound to solid material.

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