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The effects of different cations on the physicochemical characteristics of casein micelles

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Abstract

Physicochemical characterisation of casein micelles suspended in milk ultrafiltrate and enriched with different cations (Fe, Cu, Ca, Zn and Mg) was investigated. After addition of 2.5–8.0 mmol kg^{-1} of cations, associations of added cation, citrate, inorganic phosphate and calcium with casein micelles were observed. The order of association of cations with casein micelles was $Fe^{3+} > Zn^{2+} > Ca^{2+} > Cu^{2+} > Mg^{2+}$. At the same time, the casein content increased in the casein micelles while the water content decreased. Changes in hydrophobicity and zeta potential of casein micelles were also determined while no variation in the average diameter was detected. In the presence of 8.0 mmol kg^{-1} of magnesium or ferric iron, heat stability (115 °C for 30 min) of casein micelles was decreased. From these results, a mechanism of cation association with casein micelles is proposed, highlighting the determinant role of ultrafiltrable citrate and inorganic phosphate. This mechanism is discussed in relation to modifications in physicochemical characteristics of casein micelles.

Keywords: Casein micelles; Mineral; Salt; Physicochemical characteristics

1. Introduction

Enrichment of food products with various cations (especially calcium) is used to improve functional, technological and nutritional properties. In the case of dairy products, caseins which are the major proteins of milk, have an important capacity for cation binding (Gaucheron, 2004) and several studies in the literature have reported the association of calcium, magnesium, zinc, copper and iron in different milk systems. The most described systems are:

(i) Caseinophosphopeptide (Baumy, Guénot, Sinbandhit, & Brulé, 1989; Bouhallab & Léonil, 1991; Brulé, Roger, Fauquant, & Piot, 1982; Gaucheron, Mollé, Léonil, & Maubois, 1995; Vegarud, Langsrud, Rowlands, & Augustin, 2000).
(ii) Purified casein molecules in water or buffer (Baumy & Brulé, 1988; Baumy et al., 1989; Byler & Farrell, 1989; Dickson & Perkins, 1971; Emery, 1992; Harzer & Kauer, 1982; Manson & Cannon, 1978; Ono, Kaminogawa, Odagiri, & Yamauchi, 1976; Parker & Dalgleish, 1981; Reddy & Mahoney, 1991a, 1991b; Wahlgren, Dejmek, & Drakenberg, 1993).
(iii) Submicellar casein or sodium caseinate dissolved in water or buffer (Curley, Kumosinski, Unruh, & Farrell, 1998; Gaucheron, Famelart, & Le Graët, 1996a; Gaucheron, Le Graët, Boyaval, & Piot, 1997a).
(iv) Casein micelles suspended in water or buffer (Famelart, Lepesant, Gaucheron, Le Graët, & Schuck, 1996; Famelart, Le Graët, & Raulot, 1999; Le Ray et al., 1998; Lin, Leong, Dewan, Bloomfield, & Morr, 1972).
(v) Casein micelles in milk (Demott & Dincer, 1976; Gaucheron, Le Graët, Raulot, & Piot, 1997b; Green & Marshall, 1977; Hegenauer, Saltman, Ludwig, Ripley, & Ley, 1979; Jeurnink & de Kruif, 1995; Philippe, Gaucheron, Le Graët, Michel, & Garem, 2003; Singh, Flynn, & Fox, 1989a, 1989b; Udabage, McKinnon, & Augustin, 2000; Van Dijk, 1991; Van Hooydonk, Hagedoorn, & Boerrigter, 1986; Zuraw, Smietana, Szpendowski, & Chojnowski, 1986).

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However, despite these studies, it is not easy to predict the effects of cation additions on physicochemical properties of casein micelles as it is necessary to consider many factors which affect the association of cations with casein molecules. Factors include the nature and concentration of the added ion (previously, attention was mainly paid to the effect of calcium) and the composition of the milk serum (ionic composition, pH, presence or absence of whey proteins).

The aim of this study was to propose a mechanism of cation association with casein micelles suspended in the aqueous phase of milk. For this, additions of $CaCl_2$, $MgCl_2$, $ZnCl_2$, $CuCl_2$ and $FeCl_3$ were conducted on casein micelles suspended in milk ultrafiltrate. The new physicochemical properties of casein micelle suspensions (distribution of salt, protein and water, hydrophobicity, zeta potential, average diameter and heat stability) were characterised.

2. Materials and methods

2.1. Suspension of casein micelles in milk ultrafiltrate

Casein micelle suspension was prepared by dispersing phosphocaseinate powder (Schuck et al., 1994) in a milk ultrafiltrate. Milk ultrafiltrate was obtained with a 10,000 Da cut-off membrane (membrane Amicon type S10Y3, Epernon, France); 0.02% thiomersal (w/w) (Sigma, St. Louis, USA) was added to prevent bacterial growth.

2.2. Mineral solutions

$CaCl_2$,$MgCl_2$, $ZnCl_2$, $CuCl_2$ and $FeCl_3$ were purchased from Merck (Darmstadt, Germany). Stock solutions of mineral salts (0.25 mol l^{-1}) were prepared just before addition to the suspension of casein micelles. To achieve a complete dissolution of $ZnCl_2$, 1 mol l^{-1} HCl was added with agitation until haziness disappeared, i.e. 900 μl to 250 ml of stock solution. The pH values of the different stock solutions were 6.0, 5.95, 5.75, 3.80 and 1.5 for $CaCl_2$, $MgCl_2$, $ZnCl_2$, $CuCl_2$ and $FeCl_3$, respectively.

2.3. Addition of cations to casein micelle suspension and ultrafiltrate

Casein micelles suspended in a milk ultrafiltrate and ultrafiltrate alone were enriched, at room temperature, with $CaCl_2$, $MgCl_2$, $ZnCl_2$, $CuCl_2$ or $FeCl_3$ at final concentrations of 2.5, 5.5 and 8.0 mmol kg^{-1}, approximately. An appropriate amount of water was added to each sample to have the same final dilution. The suspensions and ultrafiltrates were stirred vigorously to ensure rapid and complete mixing. In parallel, a control sample (without addition of cations) was acidified to pH 6.35 with 1 mol l^{-1} HCl to reproduce the acidification caused by the addition of cations. One hour after addition of cations or HCl, pH was adjusted to 6.75 with 2 mmol l^{-1} NaOH. Samples were left standing for 24 h at room temperature with regulation of pH if necessary. The final casein concentration in the different micellar suspensions was 26.1 g kg^{-1}. Preparations of samples and subsequent analyses were carried out in duplicate.

2.4. Ultracentrifugation and ultrafiltration of casein micelle suspensions

To chararacterise the aqueous phase and the casein micelles of each suspension, ultracentrifugation at 100,000*g* (Beckman L-8-55 ultracentrifuge, Gagny, France) was performed for 1 h at 20 °C. Each supernatant was recovered and then analyzed or ultrafiltered on Ultra free 15 membranes (molecular mass cut-off: 10,000 Da) (Vivascience, Palaiseau, France).

2.5. Ultrafiltration of cation-supplemented ultrafiltrates

Cation-supplemented ultrafiltrates were ultrafiltered on Ultra free 15 membrane (molecular mass cut-off: 10,000 Da) (Vivascience, Palaiseau, France) after centrifugation at 1800*g* for 30 min.

2.6. Physicochemical characterization

2.6.1. Ion concentration

Cation concentrations (calcium, magnesium, zinc, copper and iron) in casein micelle suspensions and in milk ultrafiltrates were evaluated by atomic absorption spectrometry (Varian AA300 spectrometer, Les Ulis, France) (Brulé, Maubois, & Fauquant, 1974). Inorganic phosphate and citrate concentrations in the ultrafiltrates were determined by ion chromatography (Gaucheron, Le Graët, Piot, & Boyaval, 1996b). The experimental error for cation and anion deteminations, was ±10 mg kg^{-1}.

Concentrations in colloidal ions were deduced from the differences between total and diffusible ion concentrations; ion concentrations in ultrafiltrates were converted to diffusible ion concentrations by multiplying by a correcting factor of 0.96, in order to account for the excluded volume.

Precipitation of cations was calculated from differences between total ion concentrations in cation-supplemented milk ultrafiltrates and ultrafiltrable ion concentrations.

For each cation, ion concentration in ultrafiltrate was plotted against added cation concentration and linear regression analysis was performed to determine the slope.

2.6.2. Nitrogen content

Nitrogen content in ultracentrifugal supernatants was determined by the Kjeldahl method (IDF standard 20B, 1993). The experimental error was ±0.03 g of nitrogen per kg of ultracentrifugal supernatant.

2.6.3. Water content in ultracentrifuged pellets of casein micelle suspensions

The difference between the weights before and after drying (103 °C for 7 h) of ultracentifuged pellet (expressed as g of water per g of dry casein micelle pellet) was taken as the water content. The experimental error was ±0.02 g of water per g of dry casein micelle pellet.

2.6.4. Casein hydrophobicity

8-aniline naphtalene 1-sulphonate (ANS) is known to bind hydrophobic areas accessible to the aqueous solvent. Upon binding, its fluorescence is drastically enhanced so that exposed hydrophobic surface areas may be quantitatively determined (Bonomi, Iametti, Pagliarini, & Peri, 1988). Each suspension (0.30 ml) was diluted with its corresponding aqueous phase (obtained by ultrafiltration) to a final volume of 3.0 ml. Then, a solution of ANS (Sigma Chemical Co., St. Louis, USA) was added to a final concentration of 200 $\mu mol l^{-1}$. Spectrofluorimetric measurements were performed on a LS 50B Perkin Elmer spectrofluorimeter (Saint Quentin en Yvelines, France) at excitation and emission wavelengths of 390 and 480 nm, respectively. The emission and excitation slits were both set at 2.5 nm bandwidth. The experimental error for the relative fluorescence intensity was ±10 U.

2.6.5. Zeta potential and size of casein micelles

The zeta potential and average diameter of casein micelles were determined using a Zetasizer 3000 HS (Malvern instrument, Malvern, UK) equipped with palladium electrodes and an avalanche photodiode detector, enhancing sensitivity. Before measurement, samples were prepared by diluting 5 μl of suspension in 10 ml of its corresponding ultrafiltrate. Each filtered sample (0.80 μm filter; Pall, Chicago, USA) was measured five times at 25 °C. For determination of zeta potential, results were expressed in absolute values and the experimental error was ±0.5 mV. The experimental error for diameter determination was ±17 nm.

2.6.6. Heat stability

A heat treatment of 115 °C for 30 min was chosen to accentuate the micellar instability induced by addition of cations. For this, 10 g of suspension in a sealed pyrex tube was submerged in an oil-bath and agitated during heating. After heat treatment, samples were left standing overnight at room temperature. Heat stability was evaluated by measuring the nitrogen content (IDF standard 20B, 1993) in the filtrate (Whatman N°42 filter; Millipore, Saint Quentin en Yvelines, France).

2.6.7. Colour measurement

A microcolor tristimulus colorimeter (Minolta chromameter CR-300, 78420 Carrières-sur-Seine, France) was used for colour testing. Calibration was performed using the Minolta calibration plate (standard tristimulus values: $Y = 92.4$; $x = 0.3161$; $y = 0.3325$). In this system, L^* defines the position of the sample on the light-dark axis, a^* on the green–red axis, and b^* on the blue–yellow axis.

3. Results

3.1. Association/precipitation of added cations

3.1.1. Suspension of casein micelles

Associations of cations with casein micelles were 99%, 95%, 82%, 52% and 25% for ferric iron, zinc, calcium, copper and magnesium, respectively (Table 1).

3.1.2. Ultrafiltrate

Salt precipitations were observed for addition of each cation to ultrafiltrate (Table 2). For example, for addition of about 8.0 $mmol\,kg^{-1}$ of ferric iron, calcium, zinc, copper and magnesium, the % of cation precipitation was 92%, 91%, 79%, 51% and 48%, respectively. For the addition of calcium, magnesium and copper, the % of cation precipitation depended on the concentration of the added cation. Indeed, for additions of about 2.5 and 8.0 $mmol\,kg^{-1}$ of calcium, magnesium or copper, the % of cation precipitation changed from 23% to 91%, 27–48% and 3–51%, respectively (Table 2).

3.2. Concentration and distribution of anions

3.2.1. Suspension of casein micelles

Decreases in anion concentrations in the serum were observed for addition of the different cations. A linear relationship between ultrafiltrable inorganic phosphate and added cation was observed. Slopes of −0.68, −0.64, −0.51, −0.28 and −0.24 $mmol\,kg^{-1}$ inorganic phosphate/$mmol\,kg^{-1}$ added cation were determined for ferric iron, zinc, calcium, cooper or magnesium additions (Fig. 1(a), left). Similarly, ultrafiltrable citrate concentrations decreased for addition of cations (Fig. 1(b), left). Slopes of −0.44, −0.19, −0.08, −0.06 and −0.02 $mmol\,kg^{-1}$ citrate/$mmol\,kg^{-1}$ added cation were found for the addition of ferric iron, copper, calcium, zinc and magnesium, respectively.

3.2.2. Ultrafiltrate

Decreases in ultrafiltrable anion concentrations were also determined for addition of cations to ultrafiltrate.

Table 1
Influence of added cation concentration on cation concentration in ultrafiltrates of casein micelle suspensions and the percentage of cation association with casein micelles

Added calcium (mmol kg^{-1})	0	2.50	5.05	7.40
Ultrafiltrable calcium (mmol kg^{-1})	8.37	8.83	9.27	9.69
% of association	0	82	82	82
Added magnesium (mmol kg^{-1})	0	2.77	5.29	7.95
Ultrafiltrable magnesium (mmol kg^{-1})	2.83	4.78	6.69	8.79
% of association	0	30	27	25
Added zinc (mmol kg^{-1})	0	2.67	5.34	7.97
Ultrafiltrable zinc (mmol kg^{-1})	+	0.08	0.21	0.36
% of association	0	97	96	95
Added copper (mmol kg^{-1})	0	2.61	5.23	7.87
Ultrafiltrable copper (mmol kg^{-1})	+	0.89	2.36	3.80
% of association	0	66	55	52
Added iron (mmol kg^{-1})	0	2.87	5.52	8.03
Ultrafiltrable iron (mmol kg^{-1})	+	0.06	0.11	0.08
% of association	0	98	98	99

Table 2
Influence of added cation concentration on cation concentration in ultrafiltrates of milk ultrafiltrates and the percentage of cation precipitation

Added calcium (mmol kg^{-1})	0	2.85	5.35	7.40
Ultrafiltrable calcium (mmol kg^{-1})	8.01	10.21	8.32	8.67
% of precipitation	0	23	94	91
Added magnesium (mmol kg^{-1})	0	3.61	6.54	8.61
Ultrafiltrable magnesium (mmol kg^{-1})	3.92	6.57	7.94	8.04
% of precipitation	0	27	38	48
Added zinc (mmol kg^{-1})	0	2.57	5.28	7.87
Ultrafiltrable zinc (mmol kg^{-1})	+	0.53	1.04	1.66
% of precipitation	0	79	80	79
Added copper (mmol kg^{-1})	0	2.45	4.99	7.50
Ultrafiltrable copper (mmol kg^{-1})	+	2.38	4.77	3.69
% of precipitation	0	3	4	51
Added ferric iron (mmol kg^{-1})	0	3.10	6.43	9.57
Ultrafiltrable ferric iron (mmol kg^{-1})	+	0.29	0.40	0.72
% of precipitation	0	91	94	92

Slopes of −0.76, −0.72, −0.57, −0.18 and −0.04 mmol kg^{-1} inorganic phosphate/mmol kg^{-1} added cation were calculated for addition of ferric iron, zinc, calcium, copper or magnesium, respectively (Fig. 1(a), right). The decreases in ultrafiltrable inorganic phosphate concentrations depended on the added cation. The order of decrease was the following: $Fe^{3+} > Zn^{2+} > Ca^{2+} > Cu^{2+} > Mg^{2+}$. Slopes between −0.02 and −0.1 mmol kg^{-1} citrate/mmol kg^{-1} added cation were calculated for magnesium, copper, zinc or calcium addition to milk ultrafiltrate and −0.57 mmol kg^{-1} citrate/mmol kg^{-1} for addition of ferric iron (Fig. 1(b), right).

3.3. Concentration and distribution of calcium

3.3.1. Suspension of casein micelles

Addition of cations to suspension of casein micelles induced variations in calcium concentration in the serum (Fig. 1(c), left). Slopes of −0.32, −0.26, −0.19, −0.17 and +0.17 mmol kg^{-1} calcium/mmol kg^{-1} added cation were calculated for addition of copper, ferric iron, magnesium, zinc and calcium, respectively.

3.3.2. Ultrafiltrate

Addition of cations to ultrafiltrate decreased calcium concentration in the aqueous phase (Fig. 1(c), right). Slopes of −0.47, −0.43, −0.21 and −0.05 mmol kg^{-1} calcium/mmol kg^{-1} added cation were determined for ferric iron, zinc, copper and magnesium additions, respectively.

3.4. Concentration of soluble nitrogen

Decreases in nitrogen content in ultracentrifugal supernatants were observed for addition of copper, zinc, calcium, magnesium and ferric iron to suspension of casein micelles (Fig. 2). For example, addition of about

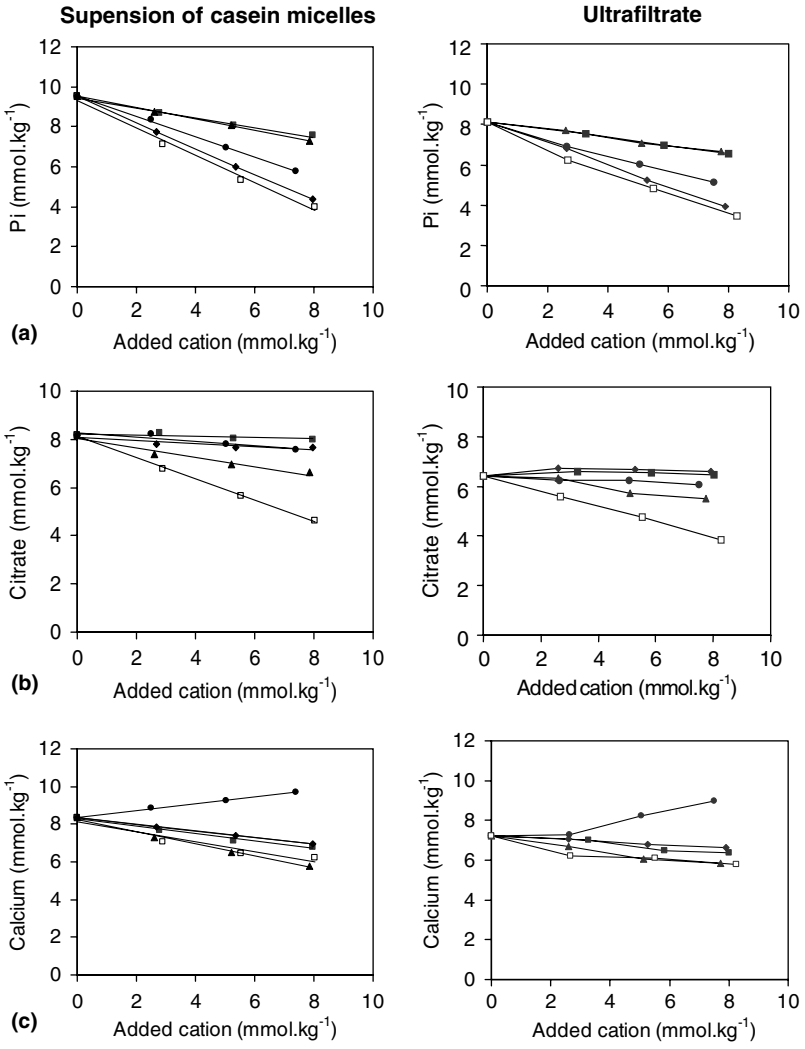

Fig. 1. Influence of added cation concentration on content of inorganic phosphate (a), citrate (b) and calcium (c) in the ultrafiltrates of different cation-supplemented casein micelle suspensions (left) and cation-supplemented milk ultrafiltrates (right). (●) calcium; (■) magnesium; (◆) zinc; (▲) copper; (□) iron (III).

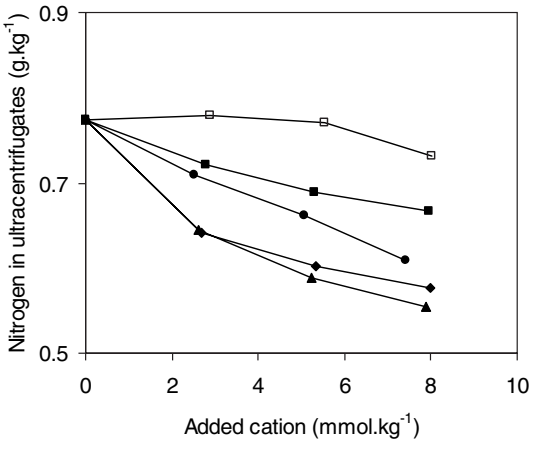

Fig. 2. Influence of added cation concentration on nitrogen content in the ultracentrifugal supernatants of different cation-supplemented casein micelle suspensions. (●) calcium; (■) magnesium; (◆) zinc; (▲) copper; (□) iron (III).

8.0 mmol kg^{-1} of cation resulted in decreases of 28%, 25%, 21%, 14% and 5%, respectively.

3.5. Water content in the ultracentrifuged pellets

After addition of cations to suspension of casein micelles, water content in the ultracentrifuged pellet decreased (Fig. 3). In the presence of 8 mmol kg^{-1} of added cation, decreases of 0.56, 0.53, 0.34, 0.28 and 0.13 g of water/g of dry pellet were found for zinc, copper, calcium, magnesium and ferric iron, respectively.

3.6. Hydrophobicity, zeta potential and size of casein micelles

Extrinsic fluorescence was relatively constant after the addition of calcium, magnesium and zinc. In contrast, addition of copper and ferric iron resulted in

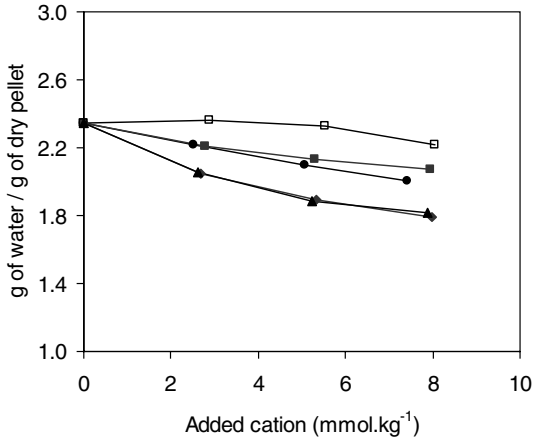

Fig. 3. Influence of added cation concentration on water content in ultracentrifuged pellet of different cation-supplemented casein micelle suspensions. (●) calcium; (■) magnesium; (◆) zinc; (▲) copper; (□) iron (III).

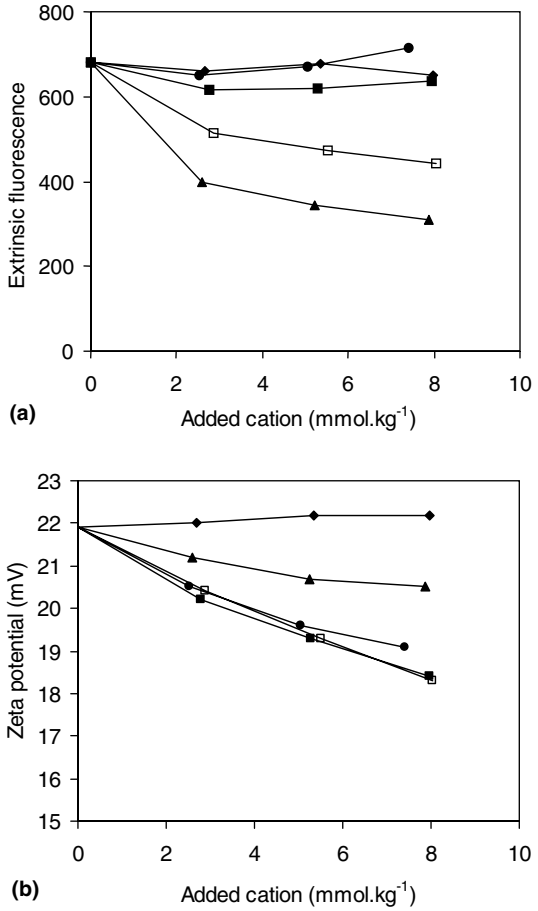

Fig. 4. Influence of added cation concentration on exposure of protein hydrophobic areas (a) and absolute value of zeta potential (b) of different cation-supplemented casein micelle suspensions. (●) calcium; (■) magnesium; (◆) zinc; (▲) copper; (□) iron (III).

decreases in fluorescence; for example, addition of about 8.0 mmol kg^{-1} induced decreases of 55% and 35% of this value, respectively (Fig. 4(a)).

After addition of about 8.0 mmol kg^{-1}, copper, calcium, magnesium and ferric iron induced decreases of −1.4, −2.8, −3.5 and −3.6 mV for the zeta potential, respectively, although in the case of zinc (Fig. 4(b)), this value was constant.

The average diameter of casein micelles stayed constant, regardless of the nature and concentration of the cation added (196 ± 17 nm).

3.7. Colour measurement of casein micelle suspensions

Addition of zinc, calcium and magnesium induced an increase in lightness, whereas a decrease was observed after addition of copper. Lightness was relatively constant after ferric iron addition (Fig. 5(a)). Green and blue hues were only affected after copper addition (Fig. 5(b) and (c), respectively). In the presence of ferric iron, the yellow hue increased (Fig. 5(c)).

3.8. Heat stability of casein micelle suspensions

The stability of casein micelles during heat treatment (115 °C for 30 min) depended on the nature and the concentration of the cation added. Addition of calcium, zinc and copper did not destabilise casein micelle suspensions. In contrast, addition of about 8.0 mmol kg^{-1} of magnesium and ferric iron resulted in decreases of 59% and 49% of nitrogen content in filtrates, respectively (Fig. 6).

4. Discussion

4.1. Modification in salt balance

From the % of cations present in the serum of cation-supplemented casein micelle suspensions, the order of association of cations with casein micelles was $Fe^{3+} > Zn^{2+} > Ca^{2+} > Cu^{2+} > Mg^{2+}$ (Table 1). At the same time, modifications in the salt balance were also determined (Table 1 and Fig. 1, left). All modifications suggest that the association of cations with casein micelles is not direct, and that citrate and inorganic phosphate in the serum play important roles in the formation of new salts (such as cation-citrate and cation-inorganic phosphate). The formation of such salts was highlighted by addition of cation to ultrafiltrate (without casein micelles) (Fig. 1, right). From cation concentrations in the serum of cation-supplemented ultrafiltrates, the order of precipitation was $Fe^{3+} > Ca^{2+} > Zn^{2+} > Cu^{2+} > Mg^{2+}$ (Table 2). In parallel with these precipitations, inorganic phosphate and citrate concentrations decreased (Fig. 1(a) and (b), right). Comparison of the results obtained after the addition of cations to casein micelle suspensions and to milk ultrafiltrate, suggests that a relationship between precipitation of

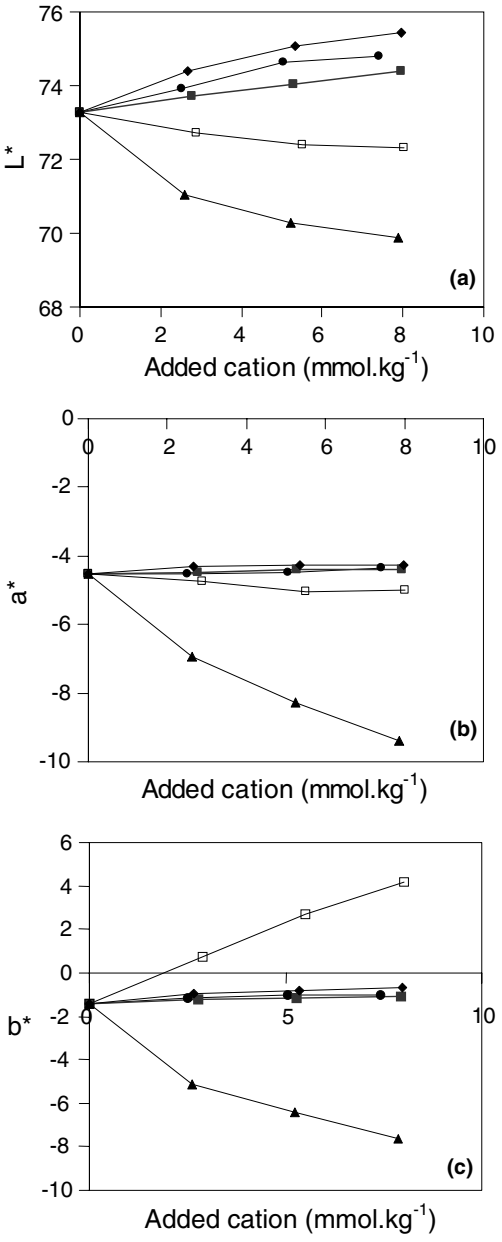

Fig. 5. Influence of added cation concentration on colour of different cation-supplemented casein micelle suspensions. Results are expressed using the L^*, a^*, b^* system where L^* defines the position of the sample on the light-dark axis (a), a^* on the green-red axis (b), b^* on the blue-yellow axis (c). (●) calcium; (■) magnesium; (◆) zinc; (▲) copper; (□) iron (III). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

newly formed salts in milk ultrafiltrate and association of added cations with casein micelles exists. Indeed, increases in the precipitation of salts in milk ultrafiltrate correspond to increases in the association percentage of added cations with casein micelles. For additions of ferric iron and magnesium, precipitations of 92% and 48% can be related to the association with casein micelles of 99% and 25%, respectively (Table 3).

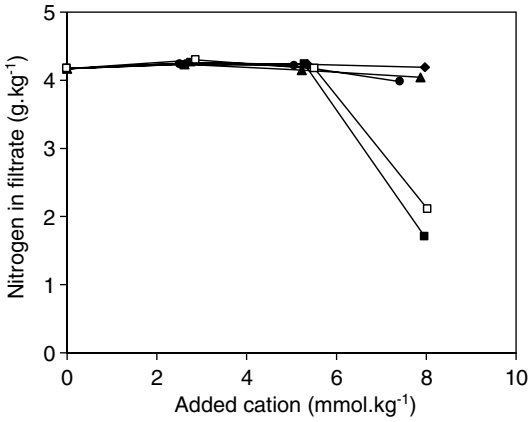

Fig. 6. Influence of added cation concentration on nitrogen concentration in filtrates after heat treatment at 115 °C for 30 min. (●) calcium; (■) magnesium; (◆) zinc; (▲) copper; (□) iron (III).

To explain the differences in association percentages of the different cations with casein micelles, several parameters must be considered. The physicochemical properties of cations, the thermodynamic constants of stability (representing the affinity between anions and cations), the solubility of the salts formed and the saturation state of the aqueous phase in these salts are essential.

Firstly, it is interesting to compare stability constants of cation-citrate and cation-inorganic phosphate with the decreases in anion concentrations in the serum and with association of cations with casein micelles. Such comparisons show that a high stability constant of cation-citrate salts (Table 3) can be related to a high decrease in the concentration of ultrafiltrable citrate (Fig. 1(b), left and right). For example, addition of about 8.0 mmol kg^{-1} of ferric iron and magnesium induced decreases in citrate concentrations of −3.5 and −0.2 mmol kg^{-1}; in these cases, the stability constants were 11.2 and 2.80, respectively. Thus, the affinity of cations for citrate could determine the amount of newly formed salts, such as cation-citrate. Similar reasoning can be applied to the formation of cation-inorganic phosphate. For example, decreases in 5.5 and 1.9 mmol kg^{-1} of ultrafiltrable inorganic phosphate concentrations were observed after addition of about 8 mmol kg^{-1} of ferric iron and magnesium, respectively (Fig. 1(a), left), and their respective constants of stability with $H_2PO_4^-$ were 3.61 and 0.60 (Table 3). However, the relation between the decrease in inorganic phosphate concentration and the constant of stability is less evident after addition of copper. In this case, minor components of milk, such as aminoacids and/or orotic acid could play an important role in the complexation of copper (Brulé & Fauquant, 1982). By keeping the copper soluble, aminoacids and orotic acid could be partly responsible for the low association percentage of copper with citrate and inorganic phosphate, and consequently with casein micelles.

Table 3
Comparison of association or precipitation of cations added to suspension of casein micelles or ultrafiltrate with constants of stability of different cation-citrate and cation-inorganic phosphate salts (25 °C, ionic strength = 0.1 mol l^{-1}) (Smith & Martell, 1976)

	Fe^{3+}	Zn^{2+}	Ca^{2+}	Cu^{2+}	Mg^{2+}
% of cation associations					
With casein micelles (cation additions of about 8.0 mmol kg^{-1} in the micellar suspension)	99	95	82	52	25
% of cation precipitations					
(cation additions of about 8.0 mmol kg^{-1} in the milk ultrafiltrate)	92	79	91	51	48
Log (stability constant) of salts					
Cation-Cit^{3-}	11.20	4.86	3.50	5.90	2.80
Cation-$H_2PO_4^-$	3.61	1.20	0.60	1.70	0.60
Cation-HPO_4^{2-}	8.13	2.40	1.30	3.30	1.80

Secondly, to explain the different modifications in the salt balance, the solubility products of the salts must be considered. The aqueous phase of milk is saturated in calcium-phosphate and calcium-citrate (Holt, 1997; Walstra & Jenness, 1984). All supplementary additions of calcium induce changes in the mineral balance, especially an association of calcium, citrate and phosphate with the micellar phase (Philippe et al., 2003). In contrast, magnesium-phosphate salt is not at saturation in the aqueous phase of milk. The low percentage of magnesium association with casein micelles (Table 1) can be explained by the low saturation indices of salts such as $MgHPO_4$ and $Mg_3(PO_4)_2$ which are below 1 (Holt, 1997). It is necessary to add more than 8 mmol kg^{-1} of magnesium to observe an association with casein micelles. For the case of copper, ferric iron or zinc additions, the same argument can be applied to explain the formation of salts such as copper-phosphate, copper-citrate, ferric iron-phosphate, ferric iron-citrate, zinc-phosphate and zinc-citrate. However, their indices of saturation are unknown and their determinations cannot be performed because solubility products and association constants are not described in the literature.

In these experiments, the distribution of calcium between aqueous and colloidal phases was also modified. Globally, the soluble calcium decreased (Fig. 1(c), left). In the absence of added cation, soluble calcium is mainly associated with citrate and inorganic phosphate and the serum is saturated in calcium phosphate and calcium citrate salts. After addition of cations, the formation of new salts, such as cation-citrate and cation-inorganic phosphate, induces a displacement of calcium and consequently an increase in ionic calcium which can interact with inorganic phosphate and citrate. Then, displacements of the equilibrium between $H_2PO_4^-$ and HPO_4^{2-} and between $citrate^{2-}$ and $citrate^{3-}$ occur. All these displacements are responsible for H^+ liberation and consequently for the decrease in pH (data not shown). Fig. 7 illustrates the different modifications in this salt balance which occur during addition of cations; the intensity of these changes depends on the nature and the concentration of the cation added.

4.2. Casein association

In the absence of added cation, suspensions of casein micelles contain a small quantity of casein molecules in the aqueous phase. This quantity is about 5% of the total casein content. In this study, addition of cations induced decreases in nitrogen content in this phase (Fig. 2). As there are no whey proteins in this study, it is probable that "free" casein molecules are also involved in cation association with casein micelles. The order of casein associations with micelles is: $Cu^{2+} > Zn^{2+} > Ca^{2+} > Mg^{2+} > Fe^{3+}$. These decreases in casein content were in agreement with a direct binding of cations to casein. These cation-casein complexes may be formed by electrostatic (Ca^{2+}, Mg^{2+} and Zn^{2+}) or coordination (Fe^{3+}, Cu^{2+}) bonds with oxygen atoms of phosphoseryl, aspartyl and glutamyl residues (Blakeborough, Salter, & Gurr, 1983; Byler & Farrell, 1989; Formicka-Kozlowska et al., 1984; Gaucheron et al., 1997a; Harzer & Kauer, 1982; Hegenauer et al., 1979; Manson & Cannon, 1978; Reddy & Mahoney, 1991a; Singh et al., 1989a, 1989b). Interactions between cations and tyrosine, histidine, phenylalanine and tryptophan residues (Dougherty, 1996) could also be implicated.

Addition of ferric iron to casein micelle suspensions showed no change in the nitrogen content in the ultracentrifugal supernatant (Fig. 2). Although, a high affinity of caseins for ferric iron was demonstrated (Brulé & Fauquant, 1982), it is probable that ferric iron is mainly associated with anions.

4.3. Modification of structure and heat stability of casein micelles

Associations of the different salts and casein molecules with casein micelles induce several changes in the micellar structure. Among these changes, hydration of

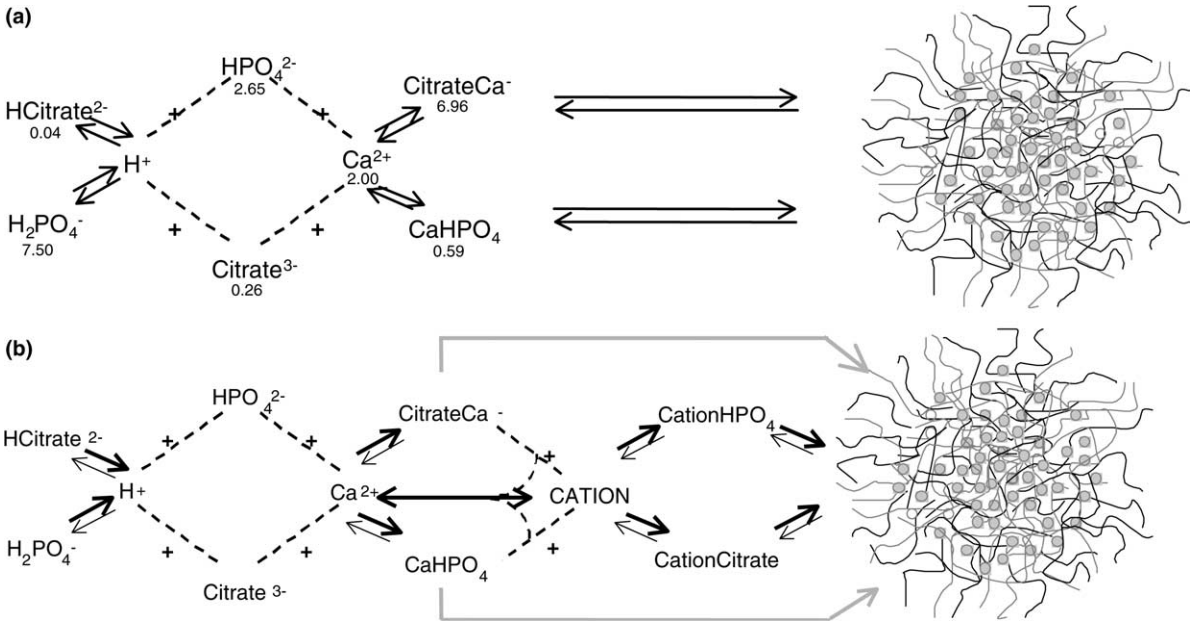

Fig. 7. Schematic representation of salt balance in casein micelles suspended in milk ultrafiltrate in the absence (a) and in the presence of cations (b). In (a), theoretical concentrations of ionic species are indicated in mmol kg^{-1} (Holt & Dalgleish, 1981).

casein micelles decreased in the presence of all cations added (Fig. 3). Similar results have been described after addition of calcium, iron and magnesium to milk (Gaucheron et al., 1997b; Le Ray et al., 1998; Philippe et al., 2003; Van Hooydonk et al., 1986). However, in spite of the decrease in hydration and increase in the contents of salts and casein molecules in casein micelles, no modifications of the average diameter of casein micelles were detected (196 ± 17 nm). These results are in good accordance with previous studies (Gaucheron et al., 1997b; Philippe et al., 2003; Udabage et al., 2000). Considering these results and results describing increases in the lightness of casein micelles in the presence of zinc, magnesium and calcium (Fig. 5(a)), it can be suggested an increase in the micellar density. After addition of copper or ferric iron, colour variations of casein micelle suspensions were due to the colour of $CuCl_2$ (blue) and $FeCl_3$ (orange) solutions (Fig. 5(b) and (c)). However, it is also probable that copper or ferric iron additions led to an increase in the micellar density.

Among the other physicochemical changes, decreases in hydrophobicity of casein micelles were also determined after addition of ferric iron or copper (Fig. 4(a)), suggesting changes in the exposition of hydrophobic segments of the casein micelles. Conversely, no changes in extrinsic fluorescence were observed after addition of zinc, magnesium or calcium (Fig. 4(a)). The concentrations of these ions were probably too low to detect changes in the accessibility of hydrophobic areas to the fluorescent probe. Indeed, in previously published studies, Philippe et al. (2003) showed an increase of 6.7% in hydrophobicity of proteins after addition of 13.5 mmol kg^{-1} of calcium chloride to milk. Gaucheron et al. (1997b) also indicated conformational modifications of casein molecules after addition of iron to milk.

Changes in the physicochemical properties of casein micelles were also deduced by comparing their zeta potential values in the absence and presence of cations (Fig. 4(b)). Decreases were observed after addition of magnesium, ferric iron, calcium or copper. Although, these decreases were not quantitatively similar, they indicate a neutralization of electronegative charges and/or a new distribution of charged aminoacid chains at the micellar surface, with possible changes in the overall thickness of the steric layer. As no zeta potentiel modification was observed after addition of zinc, we can suggest that surface modifications were too low to be detected.

As a consequence of these changes in the structure and charge of casein micelles, modifications of interactions between casein micelles were determined, especially during heat treatment. This is mainly shown by the decrease in heat stability of casein micelle suspensions containing 8.0 mmol kg^{-1} of magnesium or ferric iron (Fig. 6). Instabilities in the presence of the other cations probably exist but the concentration range studied is too low. Indeed, decreases in heat stability were determined by Jeurnink and de Kruif (1995) in the presence of 6 mmol kg^{-1} calcium-supplemented milk (140 °C for 60 min). Similarly, Le Ray et al. (1998) found instabilities of casein micelle suspensions in the presence of calcium or magnesium (19 mmol kg^{-1} added cation; 95 °C for 30 min).

5. Conclusion

This study demonstrates the important role of ultrafiltrable molecules, such as citrate and inorganic phosphate, on the mechanism of cation association with casein micelles. Formation of new salts, such as cation-citrate and cation-inorganic phosphate, depended on the affinity of the added cation for citrate and inorganic phosphate. On the other hand, the proportion of salt association with casein micelles was also determined by the mineral saturation state of the aqueous phase of milk. According to the nature and proportion of formed salts and the type of bonds between cation and casein molecules (electrostatic or coordination bonds), different structural modifications of the micellar edifice exists.

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