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pH-induced solubilization of minerals from casein micelles: influence of casein concentration and ionic strength

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Summary. The concentrations of cations (calcium and magnesium) and anions (inorganic phosphate, citrate and chloride) have been determined during the acidification of casein micelle suspensions in the pH range 6·7–1·5. The effects of casein concentration (27, 55, 83 and 144 g/kg) and ionic strength (0, 10 and 20 g/kg NaCl added) were investigated. Acidification resulted in solubilization of calcium, magnesium, inorganic phosphate and citrate ions. However, the solubilization curves were different and depended on the casein concentration. Increasing ionic strength by adding NaCl had no effect on acid-induced mineral solubilization. These results were compared with those obtained during milk acidification and discussed in relation to the mineral solubilization that occurs during curd acidification in cheese manufacture.

Acidification due to biotransformation of lactose to lactic acid is a fundamental process in the manufacture of many dairy products such as cheeses and yogurts. Most studies on the effects of acidification on minerals have been carried out with milk. However, during the acidification of milk, casein molecules are neutralized, resulting in solubilization of colloidal calcium phosphate, magnesium and citrate ions (Davies & White, 1960; Pyne & McGann, 1960; Brulé *et al.* 1974; Van Hooydonck *et al.* 1986; Visser *et al.* 1986; Dalgleish & Law, 1989; Le Graët & Brulé, 1993; Famelart *et al.* 1996; Gastaldi *et al.* 1996; Gaucheron *et al.* 1996). The results obtained with milk generally show that all the P_i and Ca^{2+} are solubilized at pH 5·2 and 4·6 respectively. In cheesemaking, acidification is mainly associated with the concentration of casein, resulting in curd draining. Depending on the cheese type, draining is carried out before (hard cooked cheeses) and/or during acidification (mould-ripened cheeses). Thus, the acidification process is carried out on curds that are more or less mineralized and more or less concentrated in casein. To our knowledge, no studies on the acidification of concentrated casein micelles have been described previously.

The aim of this investigation was to study the solubilization of minerals during pH decrease as a function of casein concentration and ionic strength. We used casein micelle suspensions in milk ultrafiltrate to obtain concentrated casein micelle suspensions in the aqueous phase of milk. To improve understanding of mineral equilibrium during curd acidification, the results were compared with those obtained with milk.

MATERIALS AND METHODS

Preparation of casein micelle suspensions

Casein micelle suspensions were prepared by dissolving native phosphocaseinate powder (Schuck *et al.* 1994; Table 1) in milk ultrafiltrate. The ultrafiltration of raw skim milk was carried out at 20 °C with a 3 kDa cut-off membrane (type S10Y3; Amicon, F-28230 Epernon, France). The final casein concentrations were 27, 55, 83 and 144 g/kg. Thimerosal (Sigma, St Louis, MO 63178, USA) was added at 0·2 g/kg to prevent bacterial and fungal growth. NaCl was added at final concentrations of 10 and 20 g/kg to portions of the sample containing 144 g casein/kg. The pH values of the samples containing NaCl at 0, 10 and 20 g/kg were 6·67, 6·51 and 6·44 respectively. All samples were stored overnight at 20 °C.

Acidification, renneting and ultrafiltration

Decreasing the pH from 6·7 to ~ 1·5 was carried out by adding 1 M-HNO_3 at 20 °C with vigorous stirring. After 2 h, rennet solution was added at 15 μg/kg to all samples to facilitate whey recovery. Samples were left standing overnight at 20 °C, centrifuged at 300 ***g*** for 5 min and the whey ultrafiltered at 800 ***g*** and 20 °C for 30 min with a 25 kDa cut-off membrane (Centriflo CF 25, Amicon). Ultrafiltrates were stored frozen at −20 °C until analyses. Ions that passed through the ultrafiltration membrane are referred to as soluble ions.

Experiments were carried out in triplicate and typical results for one experiment are shown.

Mineral analyses

Cation (Ca^{2+} and Mg^{2+}) and anion (P_i, citrate and Cl^-) concentrations in the ultrafiltrates were respectively determined using atomic absorption spectrometry (Varian, F-91941 Les Ulis, France; Brulé *et al.* 1974) and ion-exchange chromatography (Dionex, F-78354 Jouy-en-Josas, France; Gaucheron *et al.* 1996).

Calculation of ion activity products

From the mineral contents (Ca^{2+}, Mg^{2+}, Na^+, K^+, Cl^-, P_i, citrate and NO_3^-) of the ultrafiltrates, a computer program was used to calculate mineral partitions and the activities of each ion (Holt *et al.* 1981). The program was modified to include NO_3^- in the calculation, assuming that the behaviour of nitrate was close to that of chloride, so that the same constants could be used. From these activities, the apparent solubility products or ion activity products for different solid phases (brushite, octacalcium phosphate, tricalcium phosphate, hydroxyapatite and modified brushite) were calculated (Holt, 1982; Chaplin, 1984). The calculations were carried out in the pH range where calcium phosphate salts were still in the micellar phase. Thus, for a casein concentration of 27 g/kg, the pH range was 6·7–5·6, and for concentrations of 55, 83 and 144 g/kg, the range was from 6·7 to ~ 5·0. Linear regressions were calculated from the experimental results.

RESULTS

Mineral solubilization during acidification

Calcium ions. The solubilization curves for Ca^{2+} as pH decreased differed with casein concentration (Fig. 1). We found that the concentration ratio solubilized

Table 1. *Chemical composition of native phosphocaseinate powder used in this trial*

(Values are g/kg)

Dry matter	927·1
Total protein (N × 6·38)	821·9
Non-casein N	32·4
Non-protein N	3·8
Ash	77·3
Calcium	27·8
Magnesium	0·95
Inorganic phosphate	26·8
Citrate	1·5
Chloride	0·6

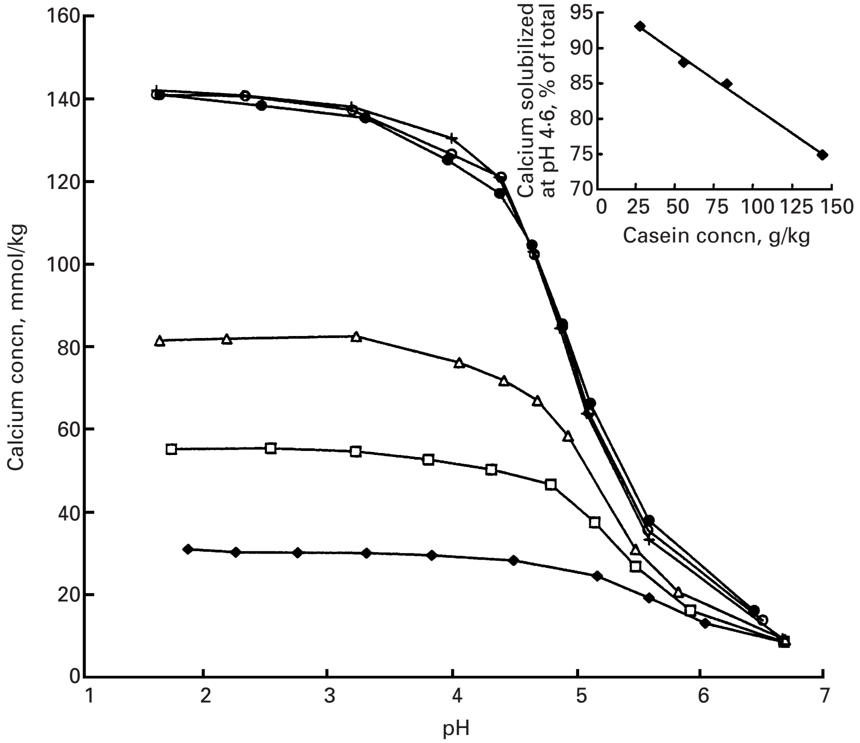

Fig. 1. Calcium concentration in the aqueous phase of casein micelle suspensions as a function of pH with casein concentrations of ◆, 27; □, 55; △, 83 and +, 144 g/kg; and with a casein concentration of 144 g/kg and NaCl added at ○, 10 and ●, 20 g/kg. Inset: calcium solubilized at pH 4·6 as a function of casein concentration; the linear regression equation is calcium solubilized = −0·1513 × casein concn + 96·94 ($r = 0·995$).

Ca^{2+} : total Ca^{2+} decreased linearly with casein concentration. As an example, the regression equation and correlation coefficient at pH 4·6 are given (Fig. 1, inset).

Adding NaCl to the casein micelle suspension containing 144 g casein/kg resulted in an increase in Ca^{2+} concentration before acidification (10·0, 14·2 and 16·5 mmol/kg for 0, 10 and 20 g NaCl/kg). During acidification, the added NaCl did not affect the Ca^{2+} solubilization and the three curves were very similar (Fig. 1).

Magnesium ions. As with Ca^{2+}, the solubilization curves of Mg^{2+} during acidification differed with casein concentration (Fig. 2). The concentration ratio solubilized Mg^{2+} : total Mg^{2+} in the aqueous phase decreased linearly as the casein concentration increased and this is illustrated for pH 4·6 in Fig. 2 (inset).

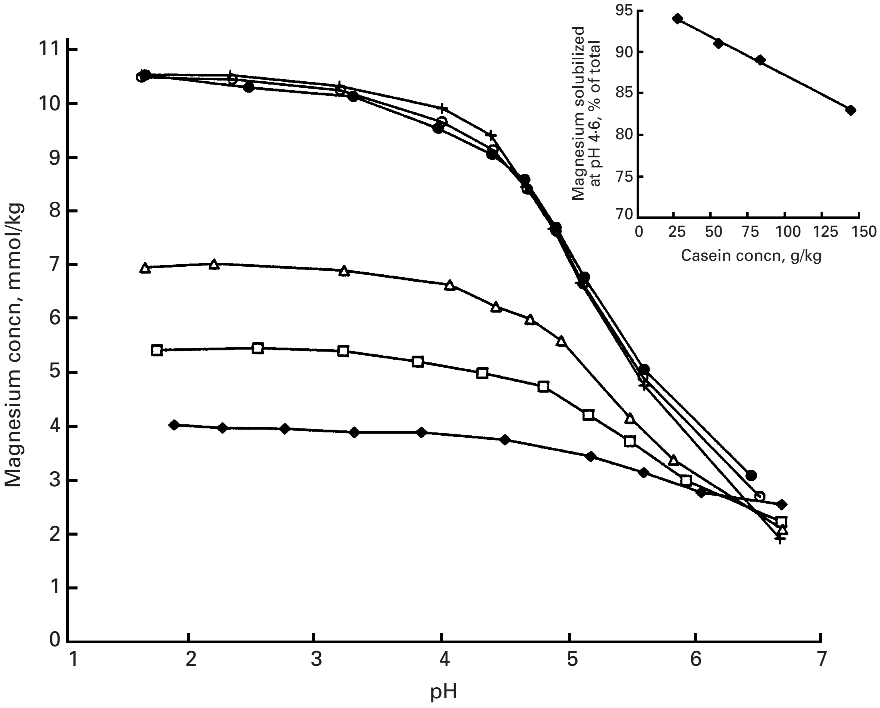

Fig. 2. Magnesium concentration in the aqueous phase of casein micelle suspensions as a function of pH with casein concentrations of ◆, 27; □, 55; △, 83 and +, 144 g/kg; and with a casein concentration of 144 g/kg and NaCl added at ○, 10 and ●, 20 g/kg. Inset: magnesium solubilized at pH 4·6 as a function of casein concentration; the linear regression equation is magnesium solubilized = −0·0927 × magnesium concn + 96·41 ($r = 0{\cdot}997$).

Addition of NaCl to a casein micelle suspension containing 144 g casein/kg resulted in an increase in Mg^{2+} concentration similar to that for Ca^{2+} (Mg^{2+} concentrations were 2, 2·8 and 3·2 mmol/kg for NaCl added at 0, 10 and 20 g/kg). As with Ca^{2+}, added NaCl did not affect Mg^{2+} solubilization during acidification (Fig. 2).

Inorganic phosphate ions. As with Ca^{2+} and Mg^{2+}, the pH-induced solubilization of P_i depended on casein concentration (Fig. 3). The maximal P_i concentrations were proportional to casein concentration. At pH 5·2, the P_i concentrations in the aqueous phase decreased linearly with casein concentration (Fig. 3, inset). At pH values between ~ 5 and ~ 3, P_i concentrations decreased only slightly. Below pH 3, there was a slight but significant increase.

The addition of NaCl to casein micelle suspensions containing 144 g casein/kg slightly increased solubilized P_i (6·9, 7·2 and 7·4 mmol/kg for 0, 10 and 20 g/kg added NaCl). However, the presence of NaCl had no effect on P_i solubilization during the acidification process (Fig. 3).

Citrate ions. Before acidification, the initial citrate concentration in the aqueous phase decreased as the casein concentration increased (Fig. 4). One explanation for this decrease can be found by considering the preparation of native phosphocaseinate powder (Schuck *et al.* 1994). During its preparation, a diafiltration step induces some release of citrate molecules initially bound to casein micelles. During reconstitution of casein micelle suspensions by dissolving the powder in ultrafiltrate, casein micelles bind part of the citrate present in the ultrafiltrate.

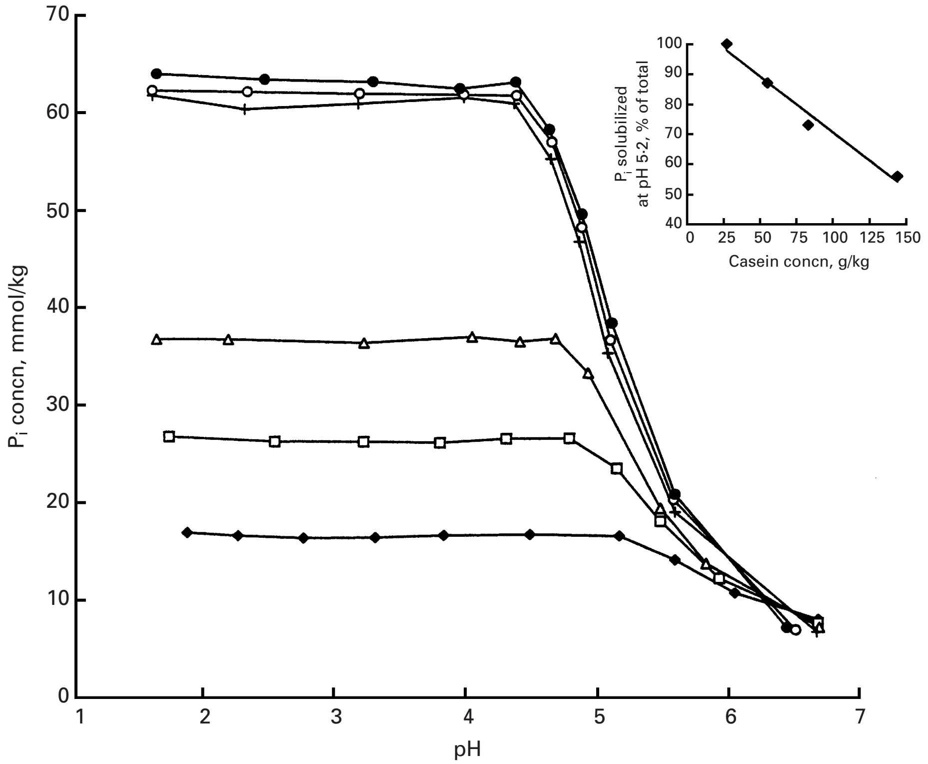

Fig. 3. Inorganic phosphate (P_i) concentration in the aqueous phase of casein micelle suspensions as a function of pH with casein concentrations of ◆, 27; □, 55; △, 83 and +, 144 g/kg; and with a casein concentration of 144 g/kg and NaCl added at ○, 10 and ●, 20 g/kg. Inset: P_i solubilized at pH 5·2 as a function of casein concentration; the linear regression equation is P_i solubilized $= -0{\cdot}3733 \times \text{casein concn} + 107{\cdot}84$ ($r = 0{\cdot}978$).

The maximal citrate ion concentrations in the aqueous phase were at pH values of about 5·15, 4·80, 4·65 and 4·10 respectively for samples with 27, 55, 83 and 144 g casein/kg. Below these pH values, the citrate ion concentration in the aqueous phase decreased until the pH reached ~ 3·0 and increased again at lower pH values (except for the casein concentration of 144 g/kg).

The addition of NaCl to casein micelle suspensions containing 144 g casein/kg resulted in a slight decrease in citrate ion concentration (5·3, 5·1 and 4·9 mmol/kg for samples with NaCl added at 0, 10 and 20 g/kg). Addition of NaCl had no major effect on the changes in citrate ion concentration in the aqueous phase during the subsequent decrease in pH (Fig. 4).

Chloride ions. During acidification, the Cl^- concentration in the aqueous phase decreased significantly (Fig. 5). Over the pH range tested, these decreases were about 10, 13, 16 and 17 % respectively of the initial concentration in the aqueous phase for suspensions containing 27, 55, 83 and 144 g casein/kg.

Before acidification, the addition of NaCl to casein micelle suspensions containing 144 g/kg in casein generally increased Cl^- concentration in the aqueous phase (31·8, 206·8 and 373·5 mmol/kg respectively for 0, 10 and 20 g/kg added NaCl). During acidification the Cl^- concentration in the aqueous phase decreased significantly (by about 17, 14 and 10 % respectively of the initial concentrations for samples with 0, 10 and 20 g NaCl/kg).

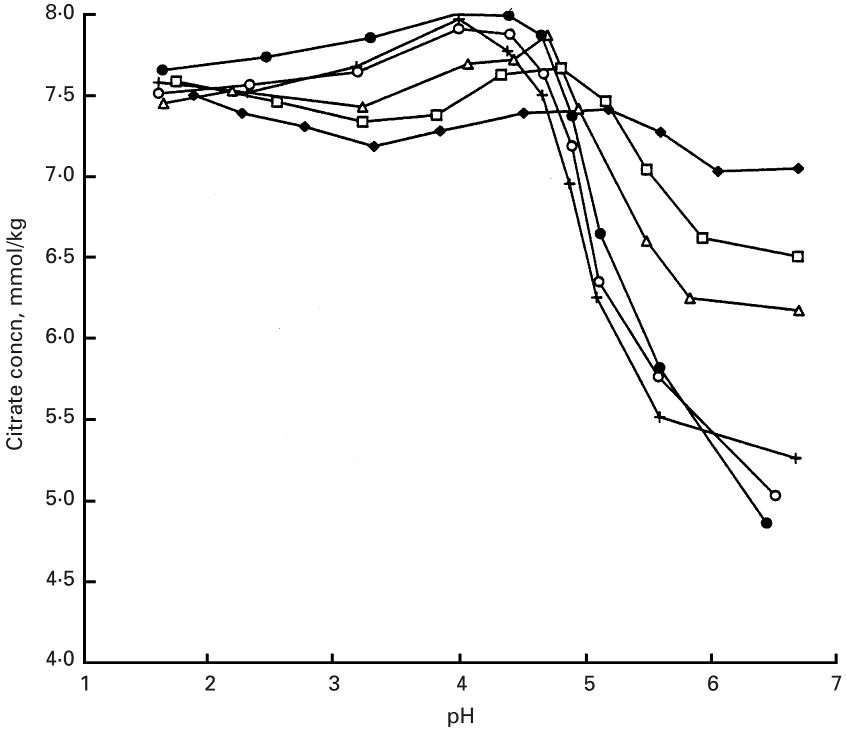

Fig. 4. Citrate concentration in the aqueous phase of casein micelle suspensions as a function of pH with casein concentrations of ◆, 27; □, 55; △, 83 and +, 144 g/kg; and with a casein concentration of 144 g/kg and NaCl added at ○, 10 and ●, 20 g/kg.

Ion activity products

The calculated log (ion activity products) values for octacalcium phosphate, tricalcium phosphate, hydroxyapatite and modified brushite were dependent on pH (results not shown). For brushite, we found that log (ion activity product) did not vary with pH (Fig. 6), in good agreement with the results obtained by Chaplin (1984) with milk. Moreover, as a function of casein concentration, the values at pH 6·7 were similar. However, calculation showed that the log (ion activity product) of brushite at pH 5·3 increased with casein concentration (Fig. 6, inset).

DISCUSSION

Influence of casein concentration on acidification-induced mineral solubilization

The pH-induced solubilization of Ca^{2+}, Mg^{2+}, P_i and citrate ions from casein micelle suspensions containing 27 g casein/kg (which corresponds to the casein content in milk) were very similar to those obtained previously with casein micelle suspensions (Famelart *et al.* 1996) and milk (Van Hooydonck *et al.* 1986; Visser *et al.* 1986; Dalgleish & Law, 1989; Le Graët & Brulé, 1993; Gastaldi *et al.* 1996; Gaucheron *et al.* 1996). These correspond to dissolution of micellar calcium phosphate. At pH values of < 4·6, the decreases in the concentrations of P_i (Fig. 3), citrate (Fig. 4) and Cl^- (Fig. 5) in the aqueous phase could be explained by these anions binding to positive charges on the casein molecules.

For more concentrated casein micelle suspensions, the acidification-induced solubilizations of Ca^{2+}, Mg^{2+}, P_i and citrate ions were generally the same as those

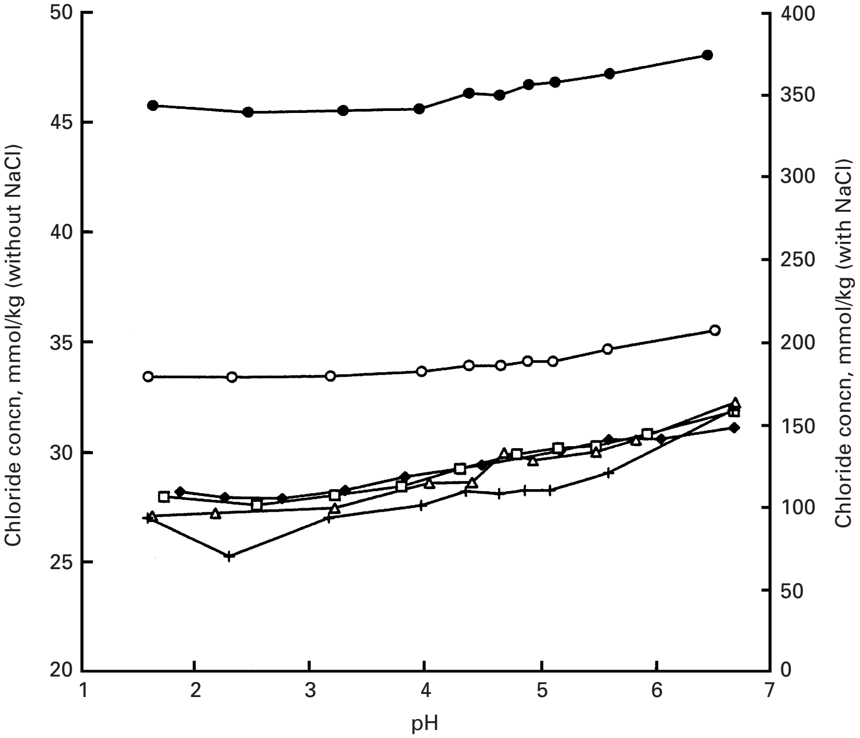

Fig. 5. Chloride concentration in the aqueous phase of casein micelle suspensions as a function of pH with casein concentrations of ◆, 27; □, 55; △, 83 and +, 144 g/kg; and with a casein concentration of 144 g/kg and NaCl added at ○, 10 and ●, 20 g/kg. The scale on the left refers to measurements without NaCl and that on the right to measurements with added NaCl.

previously observed with milk and casein micelle suspensions, but with some quantitative differences. Thus, the pH values needed for total solubilization of Ca^{2+}, Mg^{2+}, P_i and citrate ions were shifted towards lower pH with increasing casein concentration. Similar results were obtained by Gastaldi *et al.* (1997), showing that all colloidal inorganic calcium phosphate was solubilized from the casein micelles at lower pH when the total solids increased. A possible explanation of these differences is that there is a large and rapid increase in the ion activity product of calcium phosphate salts in the aqueous phase by the solubilized ions. Calculation indicates that there was an increase in the log (ion activity product) of brushite at pH 5·3 as the casein concentration increased (Fig. 6, inset). This could explain the lower mineral solubilization when the casein concentration was higher.

Solubilization at lower pH induced a shift of buffering capacity towards lower pH. Gastaldi *et al.* (1997) reported a displacement of the maximum buffering capacity from pH 5·0 to 4·8 in reconstituted milk containing 100 and 200 g total solids/kg respectively. Solubilization, especially of P_i and citrate ions, also led to an increase in buffering capacity proportional to casein concentration. Brulé *et al.* (1974) showed that the quantity of lactic acid necessary to acidify an ultrafiltration retentate was proportional to the concentration factor. Similarly, in the present study the amounts of HNO_3 needed to acidify casein micelle suspensions were higher when the casein concentration increased (results not shown). Similar observations by Gastaldi *et al.* (1997) indicated that the maximum buffering capacity is increased as the concentration of reconstituted milk increases to 100, 150 or 200 g total solids/l.

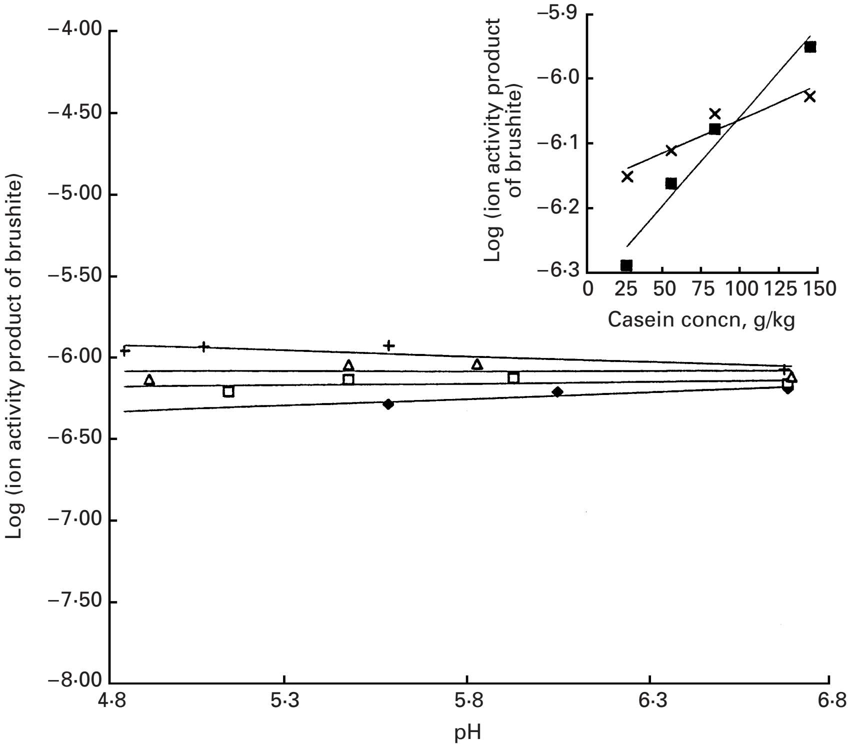

Fig. 6. Log (ion activity product) of brushite in casein micelle suspensions as a function of pH between 6·7 and ~ 5·0 with casein concentrations of ◆, 27; □, 55; △, 83 and +, 144 g/kg. Inset: log (ion activity product) of brushite as a function of casein concentration at pH values of ✕, 6·7 and ■, 5·3.

These results increase understanding of mineral behaviour during the acidification of concentrated casein micelle suspensions and can reasonably be extrapolated to acid-induced mineral solubilization in curds. They suggest that during cheesemaking, in which systems generally concentrated in protein are acidified, the role of minerals is probably underestimated for two reasons. Firstly, a significant part of the P_i was still in the colloidal phase at pH 5·2 (~ 40 % of the total for a casein concentration of 144 g/kg *v.* 0 % for a casein concentration of 27 g/kg; see Fig. 3, inset). Secondly, in contrast to the situation in milk, Ca^{2+} and Mg^{2+} were not totally solubilized at pH 4·6. At this pH value, less Ca^{2+} was released into the aqueous phase than Mg^{2+}: for a casein concentration of 144 g/kg, 75 % of the total Ca^{2+} was in the aqueous phase (Fig. 1, inset) against 83 % of the total Mg^{2+} (Fig. 2, inset). This significant difference in behaviour can be explained by the fact that the solubility products of calcium phosphate and magnesium phosphate are different (Holt, 1995). Indeed, Ca^{2+} are linked to organic and inorganic phosphate more strongly than Mg^{2+}. These ions, even at this pH value, can always participate in the structure of curds by forming bridges between two negative sites of casein molecules (e.g. organic phosphate of phosphoserine, carboxyl groups of acid amino acid).

It should be noted that the binding of P_i and Cl^- to casein molecules has some implications for the analysis of their contents in cheese. As anions bind casein molecules differently according to pH, we suggest that the pH value must be taken into account during sample preparation. Moreover, it is probable that lactate ions are also bound, although this was not investigated in the present study. However, when acidification is induced by adding HNO_3 as in this investigation, the NO_3^- are also probably able to bind to casein molecules. The citrate binding to casein molecules observed in this study is probably less important in cheese because citrate molecules are metabolized by lactic acid bacteria (Choisy *et al*. 1997).

Influence of NaCl concentration

Adding NaCl to a casein micelle suspension containing 144 g casein/kg before acidification resulted in a decrease in pH and increases in Ca^{2+}, Mg^{2+} and P_i concentrations in the aqueous phase. As observed in milk or casein micelle suspensions, these changes would correspond to exchanges of divalent cations or protons that were attached directly to phosphoseryl residues of casein molecules by Na^+ (Grufferty & Fox, 1985; Le Graët et Brulé, 1993; Famelart *et al*. 1996; Gaucheron *et al*. 1996; Le Ray *et al*. 1998). It is well known that an increase in ionic strength induces decreases in activity coefficients of the diffusible ions and consequently increases in the dissociation of the ion pairs. The slight increase in P_i concentration could be due to the pH decrease after NaCl addition.

The presence of NaCl did not strongly influence mineral solubilization during milk acidification (Le Graët & Brulé, 1993). Indeed, below a pH value of ~ 6·0 the increase in ionic strength due to NaCl addition was masked by the far greater increase in ionic strength due to solubilization of colloidal calcium phosphate. These results suggest that the increase in ionic strength associated with the brining step in cheese manufacture would have no influence on the mineral solubilization that occurs during curd acidification by lactic acid bacteria.

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