

Theoretical Model for Calculating Ionic Equilibria in Milk as a Function of pH: Comparison to Experiment

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Acidification is fundamental for the processing of milk into cheeses, caseins, and fermented dairy products. It is established that a pH decrease changes the ionic equilibria of milk, inducing the solubilization of micellar calcium phosphate. This study aimed to present a theoretical model calculating ionic equilibria in milk as a function of pH. From the pH and total concentrations of minerals and caseins, the model calculated the concentrations of all ionic species and their partition between micellar and aqueous phases of milk. As the pH decreased, the minerals present in the micellar phase were gradually displaced into the aqueous phase. The calculated concentrations of minerals were in a good agreement with the experimental ones determined from acidified milk. A very satisfactory accuracy of the calculations, estimated by a root-mean-square error (RMSE) value of 5% for Ca and P_i and a slope of the plot close to a unit, was obtained. The model is proposed for the simulation of ionic equilibria and the partition of salts between the aqueous and micellar phases of milk and dairy formulations during acidification.

KEYWORDS: Model; ionic equilibria; micellar phase; calcium; phosphate; casein; acidification; pH; buffering capacity; ionic strength

INTRODUCTION

Skim milk contains lactose, proteins (~80% caseins), minerals, and several minor components, which are distributed between the aqueous and micellar phases. At natural pH (around 6.7), the micellar phase consists of caseins and calcium phosphate, which are in physicochemical equilibrium with the aqueous phase. The mono-valents (Na, K, and Cl) are mainly present in the aqueous phase (95%), whereas Ca (70%), inorganic phosphate (P_i, 50%), Mg (40%), and citrate (Cit, 10%) are partially bound to the casein micelles (1–3). Ca is present in different forms. In the micellar phase, it is associated with P_i, forming an insoluble complex stabilized by the phosphoseryl residues of caseins (4, 5). In the aqueous phase, Ca exists as free ions (Ca²⁺, about 2 mM) or bound to anions mainly to citrate (6, 7). Ca also has a relatively high affinity for P_i, particularly the form HPO₄²⁻. However, the formation of the complex CaHPO₄ is limited by its poor solubility, responsible for the supersaturation of the aqueous phase with respect to calcium phosphate (11).

To better understand the interactions between different ions present in milk, computer programs have been proposed. They were essentially based on interactions of ions according to their respective affinities. In the early 1980s, theoretical models for the calculation of ionic equilibria, in simulated milk salt solutions or milk diffusates without proteins, had been proposed (6, 8, 9). Holt (10) proposed an equilibrium thermodynamic model of the sequestration of calcium phosphate by casein micelles and its application to the calculation of the partition of salts in milk. The calculations were based on the empirical formulas for calcium

phosphate nanoclusters assumed to be the same as the micellar calcium phosphate of milk (5). More recently, we proposed a theoretical model calculating salt equilibria in milk and mineral-enriched milk (11). The calculations were based on the interactions of ions, taking into account the solubility of calcium phosphate, the pH, and the ionic strength. From the total concentrations of minerals and phosphoseryl residues and carboxylic groups of caseins, the model calculated the salt partition between the micellar and aqueous phases of milk and milk enriched with CaCl₂, NaCl, Na₂HPO₄, or trisodium citrate (Na₃Cit). The theoretical results were in general agreement with experimental findings published in the literature (11). However, the calculations were performed at a constant pH value of 6.75, and the effect of pH was not described.

In dairy technology, pH is an important factor influencing the processing of milk into dairy products. During the acidification of milk for the manufacture of cheese, caseins, or fermented products, the pH decrease induces an alteration of ionic equilibria. The main consequences are a solubilization of minerals from the micellar to aqueous phase with a disruption of casein micelles accompanied by a precipitation of caseins at pH close to 4.6. The micellar calcium phosphate is completely depleted at around pH 5.2 (12). Indeed, at this pH value, all of the micellar P_i, citrate, and a large part of Mg are solubilized (3, 13, 14). The solubilization of micellar P_i is accompanied by a displacement of micellar calcium with a constant Ca/P_i molar ratio ranging from 1.5 to 1.9 (13, 15–19). The calcium directly bound to caseins is solubilized at low pH values (below 4.9) (17). Because the soluble P_i and citrate ions are progressively protonated during acidification, the calcium in the aqueous phase becomes free ions (Ca²⁺) (20).

However, despite all of these studies, there is still a need for theoretical models predicting the ionic equilibria in milk and

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Table 1. Chemical Composition of the Ultra-low-Heated Milk Powder

	percentage (w/w)
total solid	95.43
total nitrogen matter ^a	38.20
noncasein nitrogen ^a	9.60
nonprotein nitrogen ^a	2.80
ash ^a	8.40

^a Percentage (w/w) of dried matter.

dairy products during acidification. The objective of this study was to extend the previous model (11) to the calculation of ionic equilibria of milk as a function of pH decrease. The new parameters, the method of calculation, and the calculated data were presented. To validate the model, the calculated data were compared to experimental results obtained by acidification of reconstituted skim milk.

MATERIALS AND METHODS

Experimental Procedure.

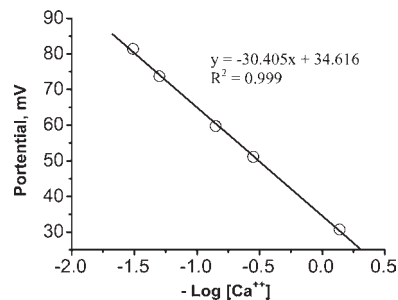
Reconstitution of Milk, Acidification, and Ultrafiltration. Milk was reconstituted from ultra-low-heated skim milk powder (Table 1), prepared as described by Schuck et al. (21), to a total solid content of 100 g kg⁻¹ using deionized water. After storing for 3 h at 25 ± 1 °C under continuous stirring, the milk was divided in 12 × 100 mL portions and acidified from initial pH (6.62 ± 0.02) to final pH (2.21 ± 0.02) by the addition of 1 M HCl. The samples were left 3 h at 25 ± 1 °C under low stirring, and the pH values were measured using a pH meter, model pH 212 microprocessor (Hanna Instruments, Romania), equipped with a pH electrode, model 90437 (Bioblock Scientist, France). To prepare the aqueous phase, 30 mL of acidified milk was centrifuged for 30 min at 1800g and 25 °C using a SV 11 TH centrifuge (Firlabo, Lyon, France) through ultra-membranes Vivaspin 20 (Vivascience, Sartorius Group, Germany), having a molecular mass cutoff of 10 kDa. Minerals that passed the membrane were referred to as soluble.

Mineral Analyses. Total and soluble concentrations of minerals were determined in milk and their respective ultrafiltrates. Concentrations of cations (Ca, Mg, Na, and K) were determined by atomic absorption spectrometry (Varian 220FS spectrometer, Les Ulis, France), and those of anions (citrate, P_i, and Cl) were determined by ion chromatography (Dionex DX 500, Dionex, Voisin-le-Bretonneux, France) (3). Analyses were carried out in triplicate, and the experimental errors were estimated to 2% for cations and 5% for anions. Total and soluble phosphorus were determined according to the FIL-IDF method (22). The concentration of micellar organic phosphate (casein-SerP, which consists of the phosphoserine residues of caseins) is calculated by subtracting the soluble phosphorus from the total phosphorus. The soluble organic ester phosphate (ester-P) concentration is deduced by subtracting soluble inorganic phosphate from soluble phosphorus.

Ionic Calcium Measurement. Ionic calcium was determined directly in acidified milk using a SevenMulti device (Mettler-Toledo, Schwerzenbach, Switzerland) fitted with a calcium-ion-selective electrode, model ISE25Ca (Radiometer Analytical SAS, France) and pH and reference electrodes (Mettler-Toledo, Schwerzenbach, Switzerland). Prior to Ca²⁺ measurement, the Ca²⁺-selective electrode was calibrated using standard solutions of CaCl₂ (1.00–50.00 mM) with 110 mM KCl as the background electrolyte. For each total concentration of CaCl₂, the Ca²⁺ concentration was calculated according to eq 1

$$C_i = \frac{a_i}{\gamma_i} \quad (1)$$

The ionic activities (a_i) for Ca²⁺ and other ionic species (i) were deduced from the resolution of the mass balance equations resulting from the potassium and calcium chloride ion pairs

**Figure 1.** Calibration of the Ca²⁺-selective membrane.

The activity coefficients (γ_i) were determined using the extended Debye–Hückel formula

$$\log(\gamma_i) = -0.5z_i^2 \frac{\sqrt{I}}{1 + 0.33A\sqrt{I}} \quad (4)$$

where A is the effective ion size parameter (nm) and I is the ionic strength (mM) calculated from the molar concentration (C_i) and the charge number (z_i) of the ion (i) (eq 5).

$$I = \frac{1}{2} \sum_{i=1}^n C_i z_i^2 \quad (5)$$

The intrinsic association constants of the interacting ions (eqs 2 and 3) and the effective ion size parameter (eq 4) were taken from Holt et al. (6). The calculation of parameters was based on successive approximations, using an iterative method as described by Mekmene et al. (11).

The electric potential measurements (mV) in the standards were plotted as a function of the logarithm of calculated Ca²⁺ concentrations (Figure 1). A total of 4 min was required for stable readings in samples. For Ca²⁺ determinations in the acidified milk, electrical potentials were measured. The calibration line was used to convert potential values to Ca²⁺ concentrations. Two series of measurements were carried out successively with two different calibrations. The experimental errors of the method of Ca²⁺ determination in acidified milk were estimated to 20%. These relatively high errors can be related to the presence of milk proteins and their pH-induced precipitation, which could cause partial fouling of the Ca²⁺-selective membrane.

Modeling. A previous paper (11) presented a model calculating salt equilibria in milk and mineral-enriched milk at constant pH 6.75. In the present study, we extended the model to calculate ionic equilibria during acidification of milk. The input data were the total concentrations of major minerals and the casein molecules represented by the binding sites of cations (Table 3). The detailed method of calculation of ionic equilibria in milk was described in previous work (11). For the acidification modeling, the same procedure was repeated at each pH value from initial pH of milk to pH around 2.2. Simulation of acidification was based on the addition of relevant protons to reach the desired pH. In the case of simulation of acidification with HCl, chloride ions are the counterions of added protons (Figure 2). The chloride concentration was adjusted to maintain the electrical neutrality obtained by summing the calculated negative and positive charges. The final value was assessed by successive approximations using a method of iterations.

The important parameters optimized for such calculations were association constants of complexes, solubility of calcium phosphate, and the Ca/P_i molar ratio of the micellar calcium phosphate.

The intrinsic association constants are presented in Table 2. Most of them were those used in the previous model (11). Certain constants have been changed to improve the agreement of theoretical results in comparison to experimental results. Thus, the association constants for the soluble organic ester phosphate (ester-P) were those for 1-Glu-P used by Holt et al. (6). The association constants for the complexes CaHPO₄ and MgHCit were taken from the tabulations of Smith and Martell (23). The association constant of the complex MgCasein-SerP was adapted from the results of Baumy (24) determined by titration of β -casein with MgCl₂ using a ³¹P nuclear magnetic resonance (NMR) method. The pK value of H₂Cit²⁻/H⁺ was that used by Upreti et al. (25). The association constants

for the complexes CaCit^- and CaCasein-SerP were determined experimentally by calcium titration of 10 mM trisodium citrate and 12 g L^{-1} β -casein, respectively. The method used for determination of calcium bindings was the Ca^{2+} -selective electrode in the presence of 100 mM KCl. The calibration of the method was carried out as described above. Apparent association constants found in the literature or determined experimentally were converted to intrinsic association constants ($I = 0$) using the extended Debye-Hückel formula as described above. The calculations were valid only for 20–25 °C because the constants used in the model were determined in this temperature range.

Another parameter that deserved our attention was the micellar calcium phosphate. Although its exact nature is still unknown, a relevant estimation of the solubility of the complex CaHPO_4 in the aqueous phase and the micellar Ca/P_i molar ratio can enable the calculation of ionic equilibria in milk. The maximum limit 0.60 mM as the concentration of the complex CaHPO_4 in the aqueous phase of milk was maintained in the present version. The micellar Ca/P_i molar ratio of 1.83 was adopted in this version. It is taken from the range of experimental data published in the literature (17, 19) and taking into account the below assumption for colloidal calcium citrate. The micellar Ca/P_i molar ratio was constant over the pH range of the study.

Assumptions were made for the presence of citrate and Mg in the micellar phase, whose exact locations are not yet elucidated. Colloidal citrate present in the micellar phase of milk was assumed to be a mixture of Ca_3Cit_2 (50%) and Mg_3Cit_2 (50%). At natural pH of milk, about 10% of citrate is in the micellar phase (1, 3). At about pH 5.20, all of the colloidal citrate is solubilized (13). In this model, it is assumed that a linear solubilization of colloidal citrate occurred between initial pH and pH 5.20.

Validation of the Model. Theoretical calculations were performed using the total concentration determined experimentally (Table 3). Dilution of milk by the addition of 1 M HCl was taken into account by multiplying the total concentrations by a dilution factor calculated for each pH value (for example, 9.20 mL of 1 M HCl was added to 100.00 mL

of milk to reach pH 2.21). First, a global comparison of the calculated and experimental results was presented. To evaluate the accuracy of predicted values, the local relative error (RE) was calculated as follows:

$$\text{RE} = 100 \times \left| \frac{C_{\text{exp}} - C_{\text{cal}}}{C_{\text{exp}}} \right| \quad (6)$$

where C_{exp} and C_{cal} are the experimental and calculated concentrations, respectively.

Second, the mineral forms (soluble Ca and P_i , total Cl, and Ca^{2+}) with wide variations of the concentrations over the pH range of the study were directly plotted. The slope and determination coefficient (R^2) were given for each mineral over the pH range of the study. The value of root-mean-square error (RMSE), expressed in percentage, was calculated according to eq 7

$$\text{RMSE} = 100 \times \sqrt{\frac{1}{n} \sum_{i=1}^n ((C_{\text{exp},i} - C_{\text{cal},i}) / C_{\text{exp},i})^2} \quad (7)$$

where n is the number of experimental values determined for each mineral over the pH range of the study. RMSE measures the dispersion of the data around the regression line. It determines the accuracy of the model, where lower values indicate less residual variance. The smaller the RMSE, the better the simulation.

Table 2. Intrinsic Association Constants (M^{-1}) Used in the Model of Calculation (6, 11, 23–25)

	H^+	Ca^{2+}	Mg^{2+}	Na^+	K^+
H_2Cit^-	1380	28	15	1	1
HCit^{2-}	6.0×10^4	876	522	10	10
Cit^{3-}	3.16×10^6	799.17×10^2	1.11×10^5	20	15.6
H_2PO_4^-	200	21	12	1	1
HPO_4^{2-}	100×10^5	542	838	16.9	13.1
PO_4^{3-}	2.47×10^{12}	2.88×10^6	8.35×10^4	100	100
Cl^-		9.4	5.6	0.92	0.92
ester- HP^-	3.19×10^6	316	295	7	6
ester- P^{2-}		10	10	1	1
casein- HSerP^-	200	11	10	1	1
casein- SerP^{2-}	2.50×10^6	6.84×10^3	6000	16.9	13.1
casein- COO^-	1.13×10^4	15	19	0.7	0.7

Table 3. Total Concentrations of Caseins and Minerals in the Reconstituted Milk (10%, w/w) at $\text{pH } 6.62 \pm 0.02^a$

components	total (mmol L^{-1}) ^c	aqueous phase (mmol L^{-1})			micellar phase (mmol L^{-1}) ^b	
		C_{exp}	C_{cal}	RE (%)	C_{exp}	C_{cal}
Ca	33.50 ± 0.15	8.04 ± 0.16	8.73	9	25.46 ± 0.31	24.77
Mg	5.25 ± 0.02	3.07 ± 0.06	3.42	11	2.18 ± 0.08	1.83
Na	18.50 ± 0.18	16.92 ± 0.34	18.22	8	1.58 ± 0.52	0.28
K	42.81 ± 0.77	39.10 ± 0.86	42.24	8	3.71 ± 1.63	0.57
Cit	9.02 ± 0.45	7.83 ± 0.39	8.12	4	1.19 ± 0.84	0.90
Cl	33.47 ± 1.67	33.16 ± 1.42	27.00	19	0.31 ± 0.25	0.00
P_i	18.80 ± 0.94	8.28 ± 0.41	7.91	4	10.52 ± 1.35	10.89
ester-P	2.37 ± 0.12	2.37 ± 0.12	2.37	0	0.00 ± 0.24	0.00
casein-SerP	7.51 ± 0.75	0.00	0.00		7.51 ± 0.75	7.51
casein-COOH	15.02^d	0.00	0.00		15.02	15.02

^a C_{exp} and C_{cal} correspond to experimental and calculated concentrations, respectively. ^bObtained by the subtraction of soluble from total concentrations. ^cExperimental concentrations also used for the simulations. ^dAssumed. Experimental values were means \pm standard deviations. RE (%) is the relative error calculated according to eq 6.

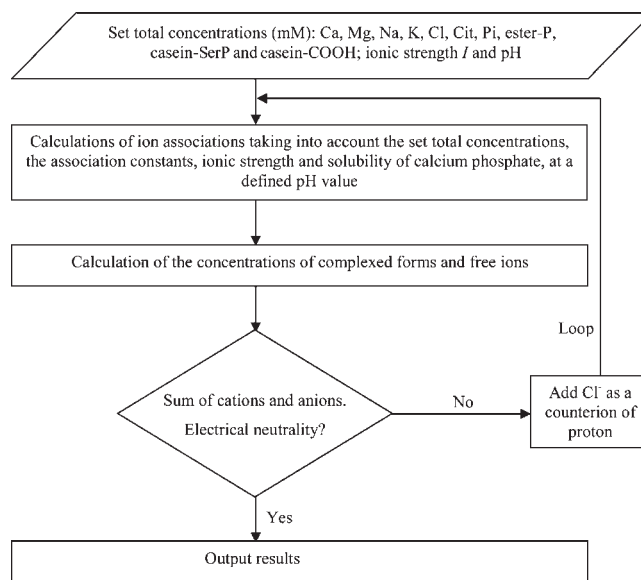


Figure 2. Simplified flowchart of the program.

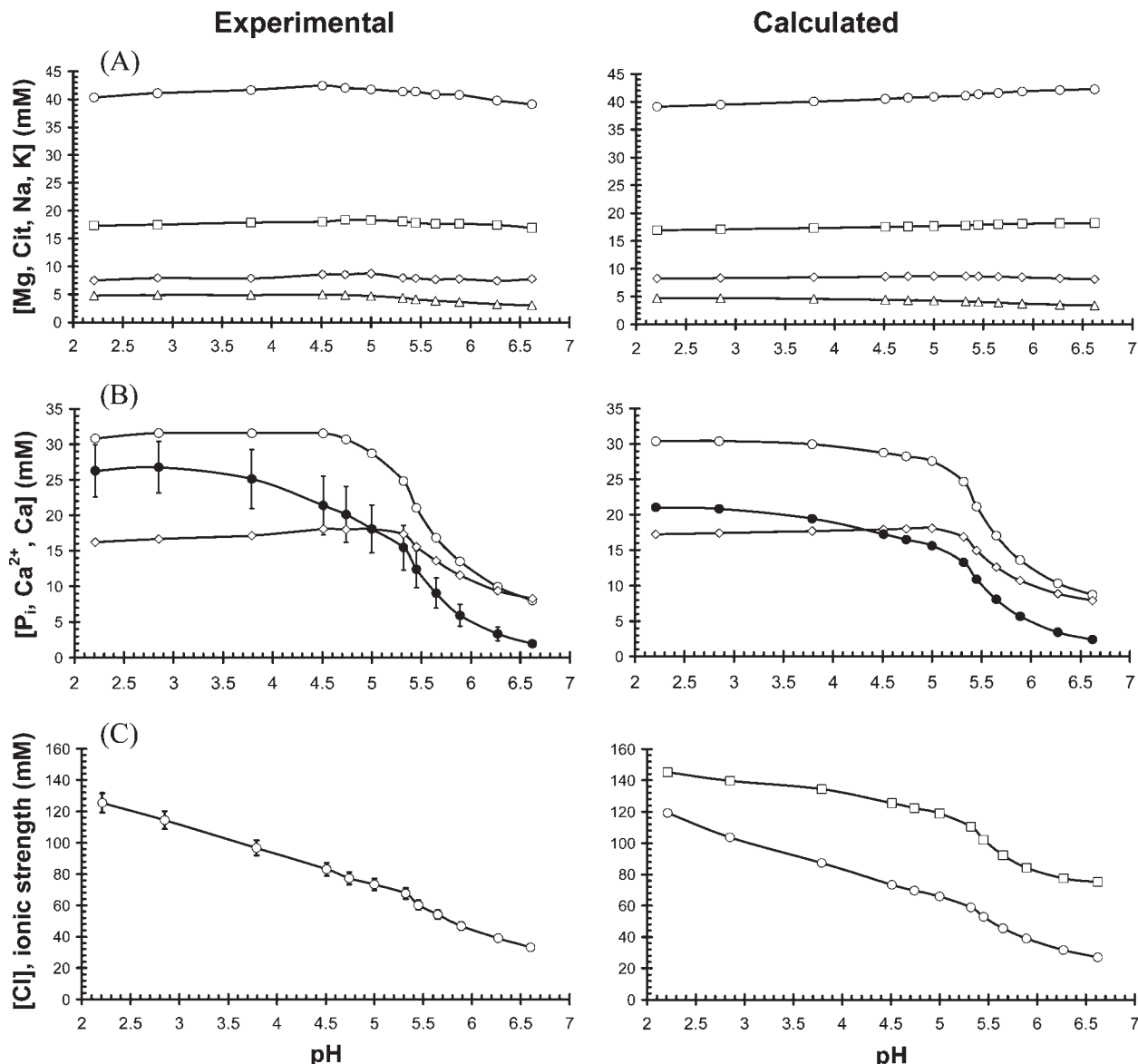


Figure 3. Experimental and calculated concentrations (mM) of soluble minerals as a function of pH. (A) Concentrations of K (○), Na (□), Cit (◇), and Mg (△). (B) Concentrations of Ca (○), P_i (◇), and ionic calcium, Ca^{2+} (●). (C) Concentration of total chloride (○) and calculated ionic strength (□).

RESULTS AND DISCUSSION

Distribution of Minerals between Aqueous and Micellar Phases at the Natural pH of Milk. Experimental and calculated mineral distributions between aqueous and micellar phases of reconstituted milk (10%, w/w) at $pH\ 6.62 \pm 0.02$ are presented in Table 3. Experimental results were in general agreement with earlier findings (2, 3, 26, 27), showing that about third of total Ca, half of total P_i , and about 90% of citrate are diffusible. Taking into account the experimental errors, the proportion of soluble P_i and citrate were in accordance with the literature, whereas that of Ca is slightly lower in our experimental results. The comparison of experimental and calculated soluble P_i , Ca, and citrate concentrations (Table 3) showed an agreement with RE of 4, 9, and 4%, respectively. About 60% of Mg is diffusible in agreement with the literature. The concentration of soluble Mg was calculated with a RE of 11%. In the same way, it is admitted that monovalents (K and Na) are mainly diffusible and only about 5% of the total concentration is in the micellar phase (13). Our experimental concentrations of non-diffusible K and Na were slightly high in comparison to the literature, and the calculated ones were clearly underestimated. The RE between experimental and calculated concentrations was

8% for both soluble Na and K. The low calculated micellar K and Na can be explained by the weak association constants of these ions for caseins used in the model. Cl ions were mainly diffusible as shown by both simulations and measurements, in accordance with the literature. However, a high RE of 19% was obtained. The case of Cl ions was unique because their concentration was theoretically adjusted for balancing the positive and negative charges. The removal of Cl ions to achieve the electric neutrality indicated that there was a deficit of positive charges resulting from the input of total concentrations of caseins and minerals. This is one of the challenges in simulating ionic equilibria in milk when some minor components or charged side chains of proteins are neglected.

Interaction of Minerals in the Aqueous Phase. The interactions of ionic species in the aqueous phase of milk are presented in Table 4. Soluble Ca was mainly associated with citrate, forming the complex $CaCit^-$ (60%), and in less degree with P_i (8%), Cl (3%), and ester-P (1%) ions. The rest (27%) was as ionic form Ca^{2+} . Calculated and experimental Ca^{2+} concentrations were 2.39 and 1.93 ± 0.44 mM, respectively. They were in agreement with other findings (28–31). Wide differences in experimental Ca^{2+} measurements were found in the literature. For example,

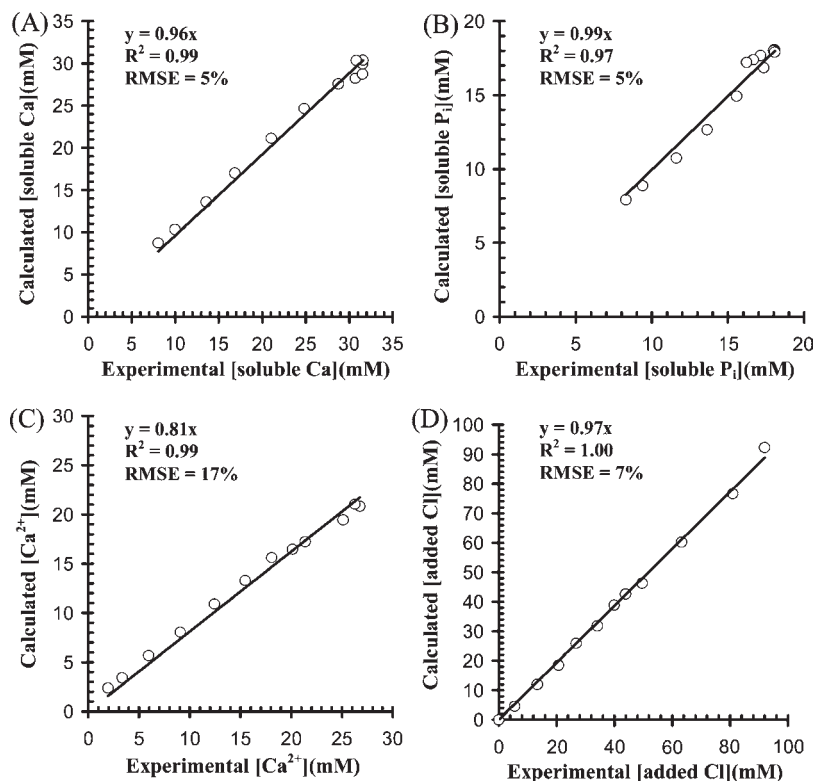


Figure 4. Comparison between experimental and calculated concentrations of (A) soluble calcium, (B) soluble inorganic phosphate (P_i), (C) ionic calcium (Ca^{2+}), and (D) total chloride (Cl). In each plot, the solid line is the regression line forced through the origin. Equations of the linear regressions, the correlation coefficients (R^2), and the RMSE values are given.

Table 4. Calculated Concentrations (mM) of Ions in the Aqueous Phase of Milk

	free	Ca^{2+}	Mg^{2+}	Na^+	K^+
H_3Cit	0.00	0.00	0.00	0.00	0.00
H_2Cit	0.00	0.00	0.00	0.00	0.00
HCit	0.10	0.03	0.01	0.01	0.02
Cit	0.36	5.20	2.30	0.04	0.06
H_3PO_4	0.00	0.00	0.00	0.00	0.00
$H_2PO_4^-$	3.22	0.07	0.01	0.04	0.08
HPO_4^{2-}	2.71	0.60	0.30	0.31	0.57
PO_4^{3-}	0.00	0.01	0.00	0.00	0.00
ester- H_2P	0.00	0.00	0.00	0.00	0.00
ester- HP^-	0.53	0.00	0.00	0.01	0.01
ester- P^{2-}	1.39	0.18	0.05	0.07	0.13
Cl^-	25.84	0.25	0.05	0.26	0.61
ionic form		2.39	0.70	17.50	40.77

values ranged from about 0.5 mM in reconstituted milk (low heat powder, 12%, w/w) to more than 3 mM in individual milk were found by Akissi-kouame et al. (32) and Holt et al. (6), respectively. Differences in Ca^{2+} concentration measurements are mainly due to difficulties related to the method of determination and the variation in milk composition.

Soluble P_i remained mainly free as $H_2PO_4^{2-}$ (41%) and HPO_4^{2-} (34%) and in less degree associated with cations. The low concentration of P_i associated with Ca in the aqueous phase is due to its low solubility. The maximum concentration of the complex $CaHPO_4$ was 0.60 mM according to the method of calculation.

Citrate ions were essentially associated with Ca as $CaCit^-$ (65%) and with Mg as $MgCit^-$ (28%), and the remainders were free (Cit^{3-} and $HCit^{2-}$) or associated with Na and K.

Mg was mainly associated with citrate (68%) and P_i (9%) or in free form (20%). The calculated concentration of free Mg^{2+}

(0.70 mM) was in agreement with the experimental and calculated values given by Holt et al. (6). Finally, ester phosphates and monovalents were mainly present as free ions.

In a general manner, the present values were in agreement with those calculated by Mekmene et al. (11). The few differences were due to the modification of the calculation parameters and the differences in the input data (pH and total concentrations) used for simulations.

Forms of Minerals in the Micellar Phase. At pH 6.62, the minerals present in the micellar phase are Ca, P_i , and small amounts of citrate, Mg, Na, and K. Micellar Ca is associated with caseins, which consist of phosphoserine residues and carboxylic groups (4.26 mM), and insoluble inorganic calcium phosphate (20.51 mM). The part of insoluble P_i in the micellar phase was 10.89 mM (about 58% of total P_i), and the parts of citrate and Mg were 0.90 mM (10%) and 1.83 mM (35%), respectively. The model calculated the micellar Ca directly bound to caseins (because of the association constants of cations toward casein-SerP and casein-COO) and the micellar Ca in the form of insoluble P_i (because of the low solubility of calcium phosphate). The calculated results quantitatively agreed with experimental results. However, the current model cannot determine the location of nondiffusible citrate and inorganic calcium phosphate in the micellar phase. Indeed, even today, the exact nature of the micellar calcium phosphate involving caseins, Ca, P_i , and small amounts of Mg and colloidal citrate is not yet elucidated. Little and Holt (5) and Holt (10) supported that the micellar calcium phosphate of milk is the same as the nanoclusters, containing Ca, Mg, P_i , citrate, and casein phosphorylated sequences called phosphate centers, which are prepared from a solution supersaturated with calcium phosphate in the presence of casein phosphopeptides. It is noteworthy that calculations were based on interactions in the aqueous phase between cations

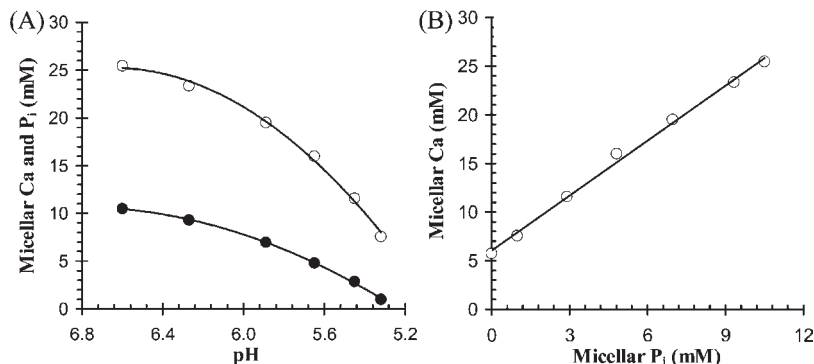


Figure 5. Experimental concentrations (mM) of micellar calcium (○) and micellar phosphate (●) during acidification of reconstituted milk (10%, w/w). The concentrations of the micellar forms are obtained by subtracting the soluble from the total concentrations of calcium and inorganic phosphate (P_i). (A) Experimental concentrations (mM) of micellar calcium (○) ($y = -9.75x^2 + 129.63x - 405.64$; $R^2 = 1.00$) and micellar phosphate (●) ($y = -4.18x^2 + 57.09x - 184.37$; $R^2 = 1.00$) during acidification of reconstituted milk (10%, w/w). Equations were given from the best fits of data. The concentrations of the micellar forms are obtained by subtracting the soluble from the total concentrations of calcium and inorganic phosphate (P_i). (B) Plots of micellar Ca versus micellar P_i ($y = 1.88x + 6.05$; $R^2 = 1.00$). The slope (1.88) gives the micellar Ca/ P_i molar ratio, and the intercept at pH 5.23 (deduced from the above equations) gives the Ca directly bound to caseins (6.05 mM).

and anions with 1:1 stoichiometry and the mixed salts were not considered.

Salt Distribution during Acidification of Milk. To study the ionic equilibria as function of pH, reconstituted milk was acidified at room temperature ($25 \pm 1^\circ\text{C}$) over the pH range of $6.62 - 2.21 \pm 0.02$ using 1 M HCl. Salt distribution between aqueous and micellar phases of milk as a function of pH was determined experimentally and theoretically using the model described above. The total concentrations of minerals and caseins (Table 2) used for calculations were corrected taking into account the effective dilution of milk induced by acid additions (for example, to reach pH 2.21 from the initial pH, milk was diluted 9%). A gradual solubilization of minerals from the micellar to aqueous phase of milk was observed during acidification. The soluble concentrations of minerals obtained experimentally and those calculated by the model are presented in Figure 3. The latter clearly demonstrates the strength of the model. Considering the extent of pH-induced solubilization of minerals, results were divided into three categories: (i) minerals mainly diffusible (Na and K) or with low pH-induced solubilization (citrate and Mg) over the pH range of the study (Figure 3A), (ii) minerals (Ca and P_i) mainly present in the micellar phase with a high pH-induced solubilization (Figure 3B), and (iii) Cl ions resulting from the acidification of milk with HCl (Figure 3C).

In the two latter cases, the experimental and calculated concentrations of soluble minerals were plotted to validate the model (Figure 4).

pH-Induced Solubilization of Na, K, Mg, and Citrate. Experimental and calculated concentrations of minerals as a function of pH showed the same trends (Figure 3A). At pH 6.62, the monovalents (Na and K) were mainly diffusible (more than 95%) and small amounts of Mg and citrate were bound to casein micelles. From pH 6.62 to about pH 5, the small amounts of nondiffusible minerals were completely solubilized. The slight decreases of the soluble mineral concentrations at low pH values were due to the dilution of milk by the addition of 1 M HCl. The experimental concentration of organic ester phosphates (ester-P), also used for calculations, was 2.37 mM and assumed to be soluble over the pH range of the study.

The monovalent cations (Na and K) of milk are mainly diffusible because the caseins preferably bind divalent ions (Ca and Mg). As showed above, the calculated concentrations of nondiffusible Na and K were low in comparison to experiments. These differences are due to the low chosen association constants between these ions and caseins (Table 3).

In the method of calculation, assumptions were performed for the colloidal citrate, which was assumed to be present as a mixture of Ca_3Cit_2 (50%) and Mg_3Cit_2 (50%). The colloidal citrate was 10% total at initial pH of milk and totally solubilized at pH 5.20. Between these two pH values, a linear solubilization of citrate was supposed, as shown by Gaucheron et al. (3). In the same way, the part of Mg bound to colloidal citrate was linearly solubilized between pH 6.62 and 5.2, and the other part of Mg directly bound to caseins was solubilized at about pH 3.

The RMSE values (eq 7) for the soluble concentrations of each mineral in the pH range of 6.62–2.21 were calculated. RMSE values of 4% were calculated for both Na and K. They were 10 and 7% for citrate and Mg, respectively. These relatively low values indicated a good accuracy of the model.

pH-Induced Solubilization of Micellar Ca and P_i . Acidification of reconstituted milk induced a solubilization of the micellar calcium phosphate as determined experimentally and calculated by the model (Figure 3B). Solubilization of micellar Ca and P_i occurred in 3 stages: from pH 6.62 to 5.23, from pH 5.23 to 4.50, and from pH 4.50 to lower pH values. In the first stage (from pH 6.62 to 5.23), solubilization of micellar calcium phosphate increased markedly with the pH decrease and was complete at pH 5.23 (Figure 5A). Indeed, at this pH value, the micellar P_i was totally solubilized. This solubilization of P_i was accompanied by a release of micellar Ca (Figure 5A) and an increase in Ca^{2+} in the aqueous phase, as shown experimentally and theoretically (Figure 3B). Experimental results of the solubilization of micellar calcium phosphate were in accordance with earlier findings (17–19, 33). The plot of micellar Ca as a function of micellar P_i in the pH range of 6.62–5.23 showed a linear relationship with a constant Ca/ P_i molar ratio of 1.88 ($R^2 = 1.00$). This ratio can be compared to that proposed by some authors. Thus, using a depletion of micellar calcium phosphate by ethylenediaminetetraacetic acid (EDTA) at constant pH, Holt (16) determined a ratio of 1.61 ± 0.04 . Using a pH-induced solubilization of minerals in milk, Chaplin (15) and Law and Leaver (18) found Ca/ P_i ratios of 1.58 ± 0.03 and 1.5–2.0, respectively. Dalgleish and Law (17) and van Hooydonk et al. (19) indicated that the ratio Ca/ P_i was about 1.7–1.9. In some studies, the phosphorus concentration determined in the aqueous phase by the colorimetric method was considered as the same as P_i , and the organic ester phosphates present in milk (34) were not taken into account. This may contribute to the difference in values of Ca/ P_i molar ratios reported in the literature.

In the second stage of solubilization (pH 5.23–4.50), micellar P_i was totally solubilized at pH 5.23 and a part of Ca remained in the micellar phase (**Figures 3B** and **5**). This part of Ca corresponded to that directly bound to caseins. At pH 5.23, its concentration was about 6.05 mM (**Figure 5B**) and the calculated concentration was 5.26 mM.

In the third stage (below pH 4.50), the almost totality of micellar Ca was solubilized. A slight decrease of the experimental concentrations of Ca and P_i was observed (**Figures 3B**). They were essentially due to the dilution of milk by the acid addition. Moreover, Le Graët and Brulé (13) supported that the decrease of solubilized P_i at low pH can be related to the adsorption of P_i ions on the acid caseins being positively charged. In calculations, only the effect of dilution was taken into account.

An increase in the concentrations of Ca^{2+} over the pH range of the study followed those of soluble Ca. Indeed, the solubilized Ca remained mainly as free ions because the principal chelating ions of Ca^{2+} (namely, P_i and citrate ions) became more and more protonated as the pH decreased. In fact, the protonated forms of citrate and P_i have low affinities to Ca^{2+} (**Table 2**). However, calculations showed a formation of a complex $CaCl^+$, which increased in the aqueous phase as the pH decreased. This association may explain why the totality of solubilized Ca was not in free form (Ca^{2+}) at low pH.

A progressive increase in the Ca^{2+} concentration as a function of pH was observed with a maximum value of about 26 ± 3.6 mM at about pH 3. The experimental concentrations of Ca^{2+} presented in **Figure 3B** were means \pm minimum and maximum values of two determinations. As reported by Parnell-Clunies et al. (20), the Ca^{2+} measurements in milk acidified to low pH values are subjected to many difficulties. In our case, the pH-induced precipitation of milk proteins might cause partial fouling of the Ca^{2+} -selective membrane. Consequently, an increase in stabilizing times was observed, particularly at very low pH values. Another possible source of errors can be related to the calibration of the electrode and the use of semi-empirical formulas for the calculation of ion activity depending upon the ionic strength (eqs **4** and **5**). However, despite the errors observed (about 20%), our experimental values seemed to be correct in comparison to the literature, where wide quantitative variations were observed (20, 32, 35). Moreover, the courses of the concentration curves during acidification described in all of these studies were very similar in shape to our results.

The plots of calculated versus experimental concentrations of solubilized Ca and P_i and Ca^{2+} showed good correlations with determination coefficients (R^2) of 0.99, 0.97, and 0.99, respectively (**Figure 4**). Slopes close to the unit were determined for solubilized Ca and P_i (0.96 and 0.99, respectively), indicating a good agreement with the experimental values. In contrast, a relatively low slope (0.81) was obtained for Ca^{2+} . This indicated an underestimated prediction of the concentration of Ca^{2+} at a pH value below 5. As discussed above, this discrepancy can be related to the difficulties encountered in the determination of Ca^{2+} by the selective membrane method in the presence of pH-induced precipitation of caseins. Consequently, the RMSE for Ca^{2+} over the pH range of 6.62–2.21 was 17%. Very satisfactory RMSE values of 5% were obtained for solubilization of Ca and P_i over the pH range of the study. This indicated the good accuracy of the model and demonstrated the relevant choice of the parameters and the method of calculations.

Increases of the Chloride Concentration and Ionic Strength during Acidification. Concentrations of total Cl and the calculated ionic strength during acidification of milk with 1 M HCl are shown in **Figure 3C**. During the acidification of milk with HCl,

various components, including caseins, whey proteins, micellar calcium phosphate, and other nonprotein materials, were titrated (36). The amounts of HCl required for titration depend upon the concentrations of these components, their ionization state, and also their form (free or bound to cations and soluble or insoluble). In other words, amounts of HCl required for the titration of milk from initial pH 6.62 to pH 2.21 are indicative of its buffering capacity. A comparison of calculated and experimental data in the acidified milk showed a good agreement (**Figure 3C**). Apart from the difference of Cl concentrations observed at initial pH 6.62 (because of the electrical neutrality requirement as explained above), the experimental and calculated concentrations of added Cl during acidification were very similar. **Figure 4D** shows the plot of calculated concentrations as a function of experimental concentrations of added Cl. A good correlation was obtained with a RMSE of 7% calculated over the pH range of the study. A slope of 0.97 indicated that the theoretical buffering capacity was slightly underestimated in comparison to the actual one. On one hand, this can be explained by the choice of the association constants and the assumptions made on the properties of micellar calcium phosphate and colloidal citrate. On the other hand, some components (charged side chains of caseins, whey proteins, and nonprotein components) contributing to the buffering capacity of milk were not considered in the model.

Figure 3C shows the calculated ionic strength (mM) as a function of pH. It was calculated from the concentrations and charges of the different ionic species present in the solution and the caseins (eq **5**). Its course during acidification mainly followed two steps. From the initial pH to about pH 5.2, the ionic strength value mainly followed the solubilization of micellar calcium phosphate. Below pH 5.2, it resulted simultaneously from the solubilization of Ca directly bound to caseins, the progressive protonation of anions, and also the increasing amounts of added Cl. The estimation of the ionic strength requires the consideration of these complex ionic equilibria. Therefore, this needs the use of the theoretical model to solve the set of equations. In dairy technology, the estimation of the ionic strength can be useful because it influences the rheological properties of acid casein gels (37) and the proteolysis of cheese (38).

In conclusion, the model presented herein calculated correctly the ionic equilibria in milk as a function of pH. From the pH and total concentrations of minerals and caseins, the model calculated the concentrations of all ionic species and their repartition between the two phases of milk. A gradual solubilization of the minerals occurred from the micellar to the aqueous phase as the pH decreased. The calculated concentrations of minerals were in a good agreement with the experimental ones determined from acidified milk. A very satisfactory accuracy of the calculations, estimated by a RMSE value (5% for Ca and P_i) and a slope close to a unit, was obtained. The model is proposed for the simulation of ionic equilibria and the partition of salts between the aqueous and micellar phases of milk and dairy formulations during acidification. A validation of the model on biological (lactic) acidification is needed.

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