

The effects of lactose on calcium phosphate precipitation

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Abstract: Calcium phosphate, as the main insoluble component of milk and dairy products, has been always of interest in dairy research. When milk is concentrated by evaporation or reverse osmosis, the calcium phosphate and lactose become supersaturated. The main objective of this work was to be able to experimentally ascertain the effect of lactose on calcium phosphate precipitation, especially at higher concentrations. The experiments were carried out at 23 °C with various concentrations of calcium and phosphate solutions without and with 9.5% w/w lactose. pH was a key factor to determine the amount of calcium phosphate precipitation. Zetasizer analysis showed that lactose strongly influenced the calcium phosphate solution by forming nanoparticles with a size of about 1 nm. The role of lactose in enabling the formation of nanoparticles was previously unknown but is likely to be an important property of milk.

Keywords: lactose, calcium phosphate, nanoparticle, precipitation.

1 Introduction

Mammalian milk and milk products are able to hold calcium and phosphate ions at concentrations over 20 times greater than their solubility in water, thus enabling the key role of carrying calcium phosphate to the infants for bone growth ([Wasserman, 1964](#); [Guéguen and Pointillart, 2000](#)). For over half a century it has been considered that lactose in milk does not influence the solubility of calcium phosphate ([Wasserman, 1964](#); [Guéguen and Pointillart, 2000](#)). Lactose is known to associate with calcium ([Herrington, 1934](#)), and possibly enhances calcium absorption in the gut ([Ghishan et al., 1982](#)), but no association with calcium phosphate is known. [Kezia et al. \(2017\)](#) found that lactose increased the solubility of calcium and phosphate ions from calcium hydrogen phosphate dehydrate (also known as dicalcium phosphate), but they did not investigate the mechanism.

Casein micelles within milk are considered the primary carrier of calcium phosphate as amorphous nanoclusters formed by interaction with κ -casein phosphopeptide ([Holt et al., 1982](#); [Holt et al., 1996](#); [Holt, 2004](#)). Lactose has not been considered to be involved at all. In an area closely related to this study, there has been considerable research into the chemistry of calcium and phosphate ions within simulated artificial body fluids (without lactose) for the biomimetic fabrication of bone-like materials ([Posner and Betts, 1975](#); [Dey et al., 2010](#); [Holt et al., 2014](#); [Park et al., 2017](#)). It is thought that ions form nanoparticles, then clusters and solid materials but the form of the nanoparticles in these systems is still under debate ([Lin and Chiu, 2017](#)).

This study was begun with research into the effect of milk minerals on “fouling” of reverse osmosis membranes used for concentration of milk ([Tew, 2015](#)) and then the effect of ion activities on solubility of calcium phosphate in milk ([Morison and Noeparvar, 2017](#); [Noeparvar and Morison, 2017](#)). Experiments with one of the simplest model solutions containing CaCl_2 , NaH_2PO_4 and NaOH showed an unexpected absence of precipitation when lactose was added. Although similar results have been obtained using simulated body fluids ([Oyane et al., 2002](#)) but no work with lactose in the calcium phosphate system has been found.

At pH less than about 6.7, H_2PO_4^- is the most abundant form of phosphate in solution. When it reaches the saturation level with Ca^{2+} it forms CaHPO_4 releasing a proton and lowering the pH. One could also state this as when an HPO_4^{2-} ion precipitates with Ca^{2+} , an H_2PO_4^- ion dissociates to HPO_4^{2-} releasing a proton. Data showing the pH change are given by [Arifuzzaman and Rohani \(2004\)](#). Thus measuring the change in pH is a simple and effective means of detection of precipitation. The appropriate solubility product, K_{sp} , is given by [Marshall and Nancollas \(1969\)](#) as 2.09×10^7 for the ion activity

product (IAP) $\{Ca^{2+}\}\{HPO_4^{2-}\}$. Saturation is defined as the ratio of IAP to K_{sp} . When the saturation is greater than 1.0 precipitation is possible (though not guaranteed).

The aim of this work was to investigate the effect of lactose on the solubility of calcium phosphate with varied and controlled pH and at different concentrations.

2 Materials and Methods

2.1 Chemicals and reagents

Fresh stock solutions of 1 mol L⁻¹ calcium chloride dihydrate (CaCl₂·2H₂O), 1 mol L⁻¹ sodium dihydrogen phosphate monohydrate (NaH₂PO₄·H₂O), and sodium hydroxide (NaOH). Solutions of monohydrate lactose (C₁₂H₂₂O₁₁·H₂O) with a concentration of 10% w/w were made up for experiments containing lactose. For all experiments, ultrapure water was deionised by a Milli-Q® integral water purification system which was taken freshly by a Q-POD® ultra-pure water remote disperser (Merk Millipore, USA).

All experiments were carried out in a laboratory that was always maintained at 23±1.0°C. All glassware and containers were rinsed with Milli-Q water. All micropipettes were calibrated accurately for avoiding any possible measurement errors by weighing Milli-Q water using a balance with a resolution of 0.1 mg.

2.2 Preparation of calcium chloride and sodium phosphate solutions

For each mixed solution, solutions of both calcium chloride and sodium phosphate (unmixed) were prepared in a 100 mL volumetric flask with various concentrations, and sodium hydroxide solution was added to achieve a desired pH values.

The calcium chloride and sodium phosphate solutions were transferred individually to 250 mL plastic containers, lids of which had holes for gas and probes, and then they were stirred with a magnetic stirrer. Instrument grade argon gas was bubbled at 40 cm³ min⁻¹ to the bottom of each solution to strip any carbon dioxide and prevent absorption which strongly influenced pH. Once the individual solutions had reached a stable pH they were mixed. pH, conductivity, and temperature were measured and recorded over the time with argon still bubbling. Samples were taken from time to time for particle size analysis using a Zetasizer to identify the possible formation of nanoparticles.

2.3 Preparation of calcium chloride, sodium phosphate, and lactose solutions

Two 9.5% w/w lactose solutions were prepared, by adding 10% lactose monohydrate to water, and incubated at 40°C with orbital shaking at 140 rpm for approximately 4 hours to ensure complete dissolution of lactose. They were then stored at laboratory temperature overnight. One lactose solution was used for preparation of calcium chloride solution and the other one for sodium phosphate solution, each of which were made up and mixed similar to the procedure for without lactose solution.

2.4 Analytical instruments

The pH of solutions was measured by a pH meter that was equipped with automatic temperature compensation. The probe and meter were calibrated before each set of measurements using three standard buffer solutions with pH values of 4, 7, and 10.

The electrical conductivity of the solutions were measured with a Lab 960 conductivity meter that was equipped with a Lab LF413T standard conductivity cell (Schott, Germany). The conductivity meter was set to a linear temperature compensation with an accuracy of ±0.5% and a temperature coefficient of 1.754 % K⁻¹.

The Zetasizer (Model Nano Series, Malvern, United Kingdom) was used to determine size of particles in the mixed solutions. Three run were defined in the software, in which water was chosen as dispersant with refractive index of 1.333 and absorption of zero. Temperature and equilibrium time were set to 23°C and 90 minutes, respectively.

3 Results and Discussion

3.1 Titrations of CaCl₂ and lactose solutions

In initial experiments without argon it was found that the pH of ingredient solutions, especially CaCl₂, changed rapidly (within minutes) when magnetically stirred and exposed to air. Titration results with NaOH were inconsistent as shown in Figure 1, and clearly showed absorption of CO₂ during a lunch break. The equilibrium pH of CaCl₂ before the addition of NaOH was less than 5.5.

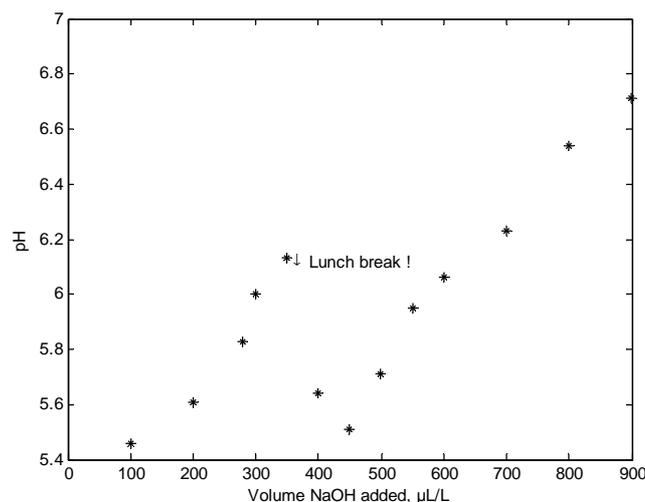


Figure 1: Titration of 60 mM CaCl_2 solution with NaOH while stirred and open to air

When argon was bubbled through the solution, the pH values were about 1.5 units higher and more repeatable between experiments. Solutions of sodium phosphate were much more stable but these were argon gassed also to avoid CO_2 absorption. It was then possible to obtain solutions with a stable desired pH.

During pH adjustment it was noticed that adjustment of lactose solutions required more NaOH than expected. Lactose can dissociate a very small amount in solution with a K_a given by [Stern \(1931\)](#) of 7.6×10^{-13} . Using this acidity constant one can calculate that a 9.5% solution has an expected pH of 6.33. Once 3 mmol/L of NaOH has been added the pH is expected to tend to about 11.5 but the pH reached only 10.2 in the experiments. The test was repeated using four different samples of lactose, both from New Zealand dairy companies and also from the laboratory chemical supplier BHD Chemicals, though it is possible that all samples had the same origin. The possibility of the presence of lactose phosphate was considered as this would have the effect of lowering the pH and would interact with NaOH more than lactose ([Lifran, 2007](#)), but much more investigation is required to eliminate this source of uncertainty.

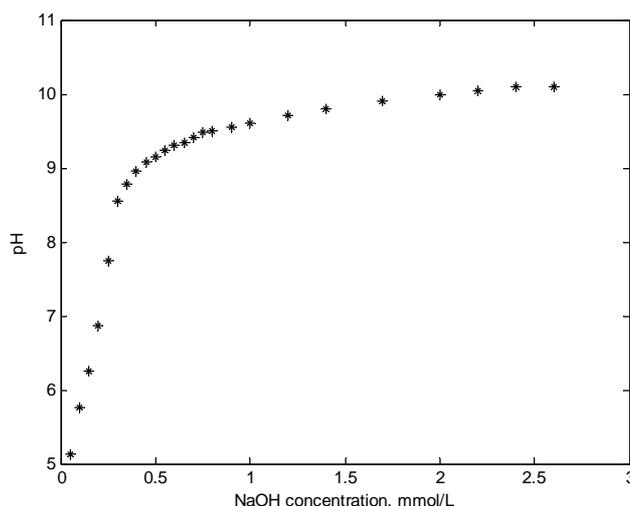


Figure 2: Titration of 9.5% lactose solution with NaOH

3.2 Effect of lactose on calcium phosphate solution

The speciation of calcium and phosphate in solution was calculated using MSA theory to determine activity coefficients ([Morison and Noeparvar, 2017](#); [Noeparvar and Morison, 2017](#)). Typical results for phosphate are shown in Figure 3a and for Ca^{2+} in Figure 3b. These calculations are done with the assumption that all products remain as single ion pairs in solution and do not precipitate.

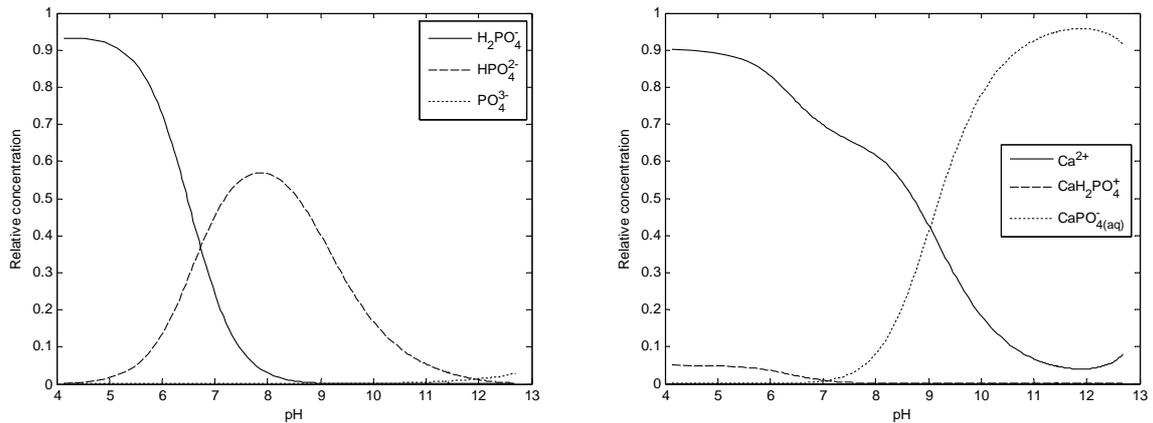


Figure 3: Relative concentrations of 5 mM a) phosphate ions and b) calcium ions and compounds in water.

From the calculation of ion speciation and activities one can obtain the ion activity product and hence the saturation as shown in Figure 4. Dynamic calculation have also been performed, but not shown here, that show the progression of precipitation. The concentration of both Ca^{2+} and PO_4^{3-} in milk serum are both about 10 mM (Walstra et al., 2006). If no other ions were present, which would reduce the ion activities, then 10 mM Ca^{2+} and PO_4^{3-} would be about 25 times the saturation limit at a typical milk pH of 6.7.

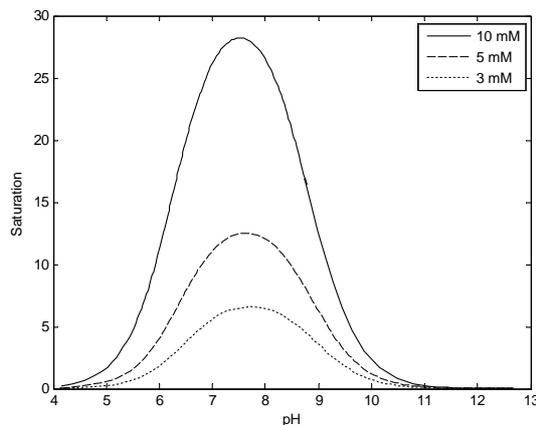


Figure 4: Saturation of CaHPO_4 at different molar concentrations of Ca^{2+} and PO_4^{3-}

A range of pH values and concentrations with a 1:1 ratio of Ca:P were tested. At 2.5 mM (Ca^{2+} and PO_4^{3-}) and pH 6.7, no precipitation was detected either by eye, by shining a laser pointer through the solution or using the Zetasizer. At lower pH values, solutions with higher concentrations did not precipitate, e.g., 10 mM with or without lactose at pH 5.2 did not precipitate. This is consistent with the lower saturation at lower pH seen in Figure 4.

Differences were seen for 5 mM solutions with and without lactose at pH 6.5 to 6.3 (Figure 5a). In this figure, it can be seen that precipitation, as indicated by the pH drop, is not instantaneous. This is explained by the rate of precipitation being proportional to the area of precipitated particles already in the system. The solution with lactose delayed precipitation even though it was at a slightly higher pH and hence less stable. Similarly, for 3.5 mM (Figure 5b) with almost identical pH in both solutions, the presence of lactose increased the stability of the solution. No precipitation was observed even after 200 minutes. In future experiments the long term stability of this and other solutions will be assessed.

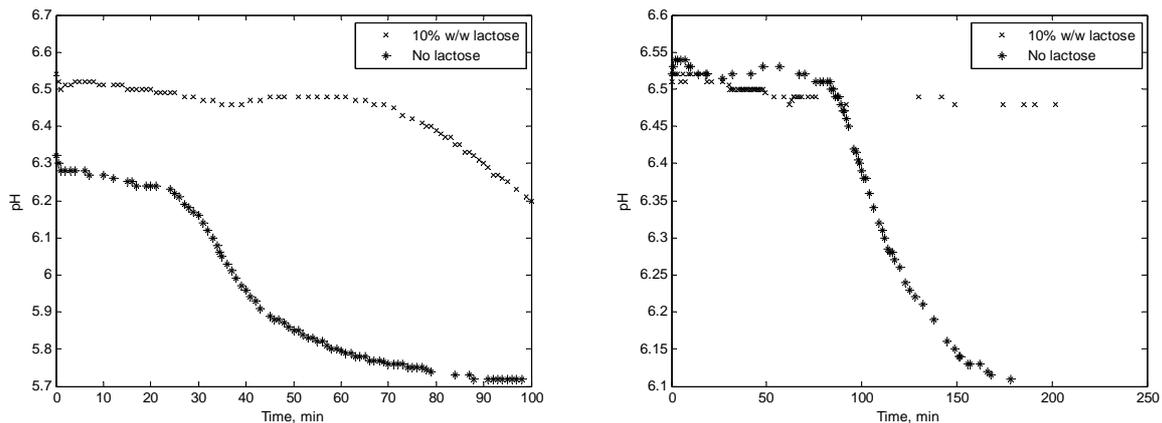


Figure 5: pH changes, indicating precipitation, for a) 5.0 mM and b) 3.5 mM CaCl_2 and NaH_2PO_4 with and without lactose.

The most striking difference between solutions with and without lactose was shown by the Zetasizer response as seen in Figure 6. At least 10 different mixtures with lactose at a pH less than 7 all showed a peak at a particle diameter close to 1 nm. Mixtures without lactose did not show this peak. The same peak was observed in a sample with lactose that had been kept for 3 days before particle size measurement, indicating that the particles were stable. Samples of lactose only did not show any peaks so it was concluded that the peak is from a reaction product and not an artefact.

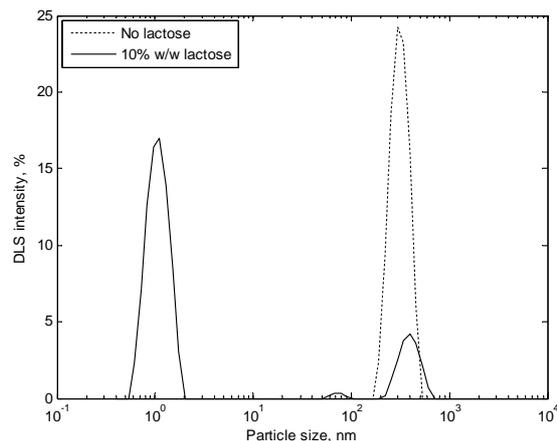


Figure 6: Zetasizer responses for mixtures of CaCl_2 and NaH_2PO_4 with and without lactose.

Similar nm size particles have been found in simulated body fluids by [Oyane et al. \(2002\)](#) using 2.5 mM Ca^{2+} and 1.0 mM HPO_4^{2-} at pH 7.4 and by [Onuma and Ito \(1998\)](#). [Kellermeier et al. \(2012\)](#) found stable clusters in a calcium carbonate silica system. A number of structures have been proposed. A popular one has been the Posner cluster ([Posner and Betts, 1975](#)) $\text{Ca}_9(\text{PO}_4)_6$ which has a theoretical diameter of 0.95 nm. [Lin and Chiu \(2017\)](#) simulated possible structures for calcium phosphate clusters with sizes of about 1 nm and obtained some alternative structures with lower energies than the Posner cluster.

The result shown in Figure 6 is the key result of this work. When lactose is added to the calcium phosphate system with concentrations similar to those found in milk, nanoparticles are formed. The formation of these seems to prevent or reduce the precipitation of calcium phosphate and hence will add to the stability of calcium phosphate in milk. This effect will be in addition to the role of casein in stabilising calcium phosphate in milk as discussed by [Holt \(2004\)](#). Lactose will act in the milk serum (outside of the casein micelles) so might have a role in stabilising the fraction in the serum.

There are many uncertainties in these experiments and the mechanism for the increased stabilisation is unclear. It is reasonably certain that when lactose is present, nanoparticles of about 1 nm are formed.

There is no evidence at this stage of the composition or structure of these particles. Given the unexpected titration curve of lactose, it is possible that there is a significant amount of lactose phosphate or other substance in the lactose and that this forms part of the nanoparticle. If lactose does not make up part of the nanoparticle, there are other mechanisms for interactions. The presence of lactose will increase the solution viscosity and possibly reduce the rate of normal precipitation. Disaccharides like sucrose, maltose, and lactose typically are hydrated by about five water molecules in solution, which reduces the water activity, hence affecting calcium and phosphate ion interaction. [Little and Holt \(2004\)](#) showed that β -casein phosphopeptide in milk will stabilise casein phosphate nanoparticles, but did not speculate on a structure. Likewise [Oyane et al. \(2002\)](#) who measured 1 nm calcium phosphate particles did not discuss their structure. [Lin and Chiu \(2017\)](#) showed that the structures have not yet been confirmed.

In this preliminary work the Ca:P ratio used as 1:1, but the Posner cluster has a ratio of 1.5:1. [Little and Holt \(2004\)](#) suggested a lower limit of 1.15. It is expected that a ratio closer to 1.5 will increase stability so some future experiments will be carried out with this ratio.

The nanoparticles have been found to be stable for at least 3 days, but this duration needs to be extended and tested for a range of conditions. [Oyane et al. \(2002\)](#) showed stability for 7 days in simulated body fluids.

Lactose is the first sugar found to form nanoparticles with calcium phosphate. The disaccharides and monosaccharides need to be tested to see how specific this effect is.

3 Conclusions

The experimental results clearly show the formation of a 1 nm nanoparticle in solutions of lactose and calcium and phosphate ions. Lactose was found to prevent or slow down the precipitation of calcium phosphate, probably as calcium dihydrogen phosphate. The structure of the nanoparticle is not known and, based on current research in this area, might be difficult to determine. The effects of many of the conditions such as concentration, ion ratios, pH, lactose concentration, and time need to be tested.

The effects of lactose in this ideal system indicate that it might have a role for the stabilisation of calcium phosphate in milk that might add to the stabilisation produced by the casein micelles.

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